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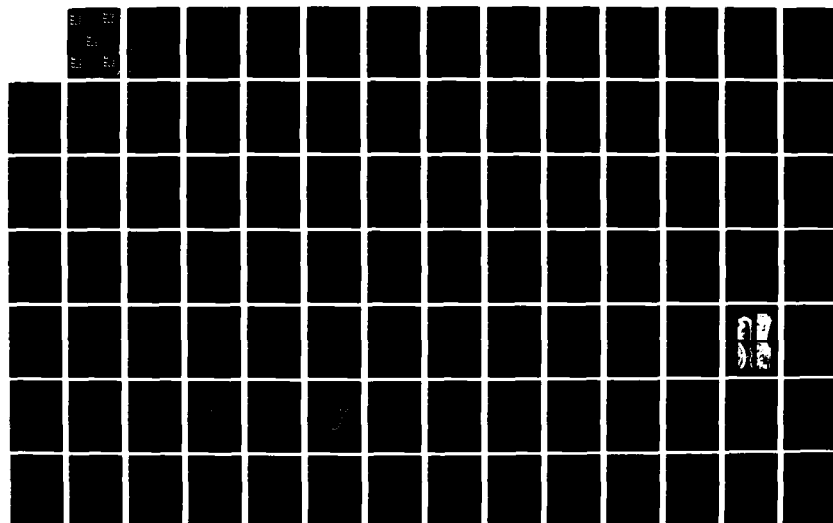
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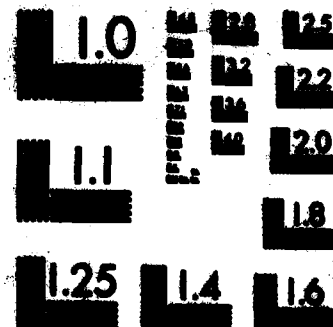
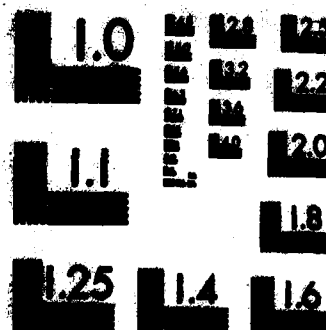
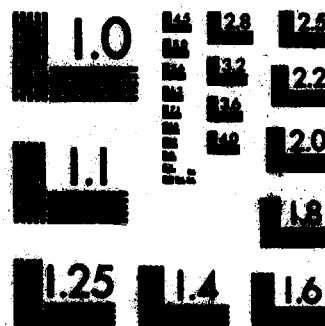
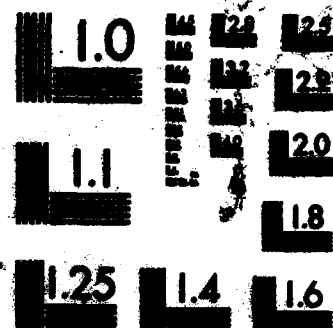
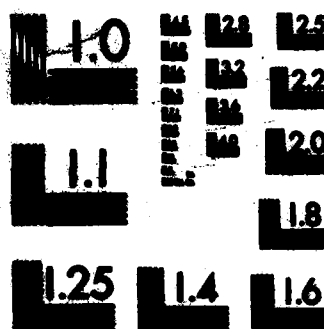
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THE MECHANISMS OF ADHESION OF ENTEROMORPHA
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John Campbell III

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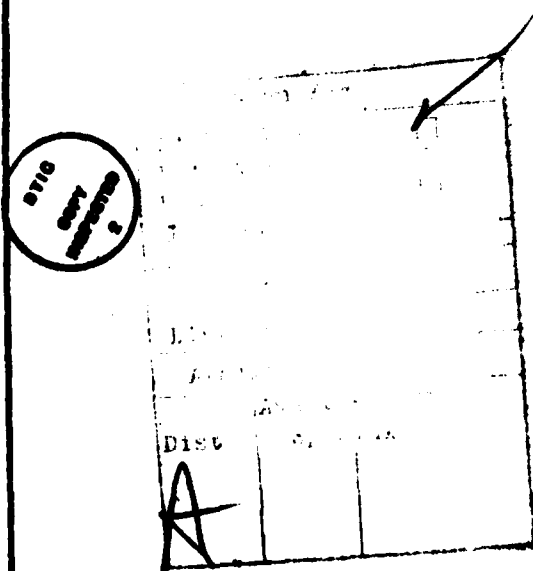
and surface. Unlike bacterial attachment, E. clathrata was found to attach to a wide variety of surfaces including surfaces with absorbed proteins of a wide range of isoelectric points.

As the cells began to grow and develop rhizoidal filaments, they produced an extracellular polymer. This polymer was composed of carbohydrate and protein. This extracellular polymer (adhesive material) allowed the cells to bridge the gap separating them from the surface. The cells of E. clathrata were then in direct molecular contact with the surface which was called the second phase of attachment (semi-reversible adhesion). The extracellular polymer produced a stronger attachment than in the first phase of attachment but the adhesion was still weak to any shear force.

As the pH of the medium increased, cross-linking began to occur between the individual polymer chains. These cross-links were ionic in nature between calcium and probably fucose and carboxyl groups of uronic acid derivatives. As the number of cross-linkings increased, the viscosity of the extracellular polymer increased to a point where attachment became very strong. The cells were now in the third phase of attachment (irreversible adhesion).

Factors that caused a loss of irreversible adhesion such as lowering the pH, darkness, DCMU, and metal chelators were all shown to do so by causing a release of the calcium from the extracellular material.

The extracellular material responsible for adhesion was composed of carbohydrate and protein at a ratio of 23:1. The carbohydrate portion of the adhesive material was made up of fucose, galactose, glucose, and uronic acid derivatives.



ABSTRACT

→ The mechanism of attachment of Enteromorpha clathrata was determined. The attachment process consisted of three phases. In the first phase (reversible adhesion) cells of E. clathrata were held weakly to a negative surface at a point called the secondary minimum where the forces of attraction (van der Waal) and repulsion (electrostatic) were in equilibrium. The cells were prevented from closer contact and subsequent stronger attachment by the strong primary repulsive forces. When the surface was a hydrophobic one, then this initial attraction was due to hydrophobic interaction on the part of cell and surface. Unlike bacterial attachment, E. clathrata was found to attach to a wide variety of surfaces including surfaces with absorbed proteins of a wide range of isoelectric points.

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ABBREVIATIONS

DCMU 3-(3,4-Dichloropheney)-1,1-dimethylurea

HOEDTA Chel DM Acid

EDTA Ethylenediaminetetraacetic acid

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INTRODUCTION

The attachment and subsequent growth of bacterial, plant, and animal organisms on the hulls of ships and other submerged marine structures is known as fouling (97). Fouling is a natural process involving competition and succession. This process has important economic implications when the cumulative effects become deleterious to the functioning of a structure. This situation constitutes the problem of aquatic fouling which is of particular importance in the marine environment (15).

The problems of marine fouling have plagued mankind for as long as the oceans have been used, with written records as early as the fifth century B.C. (9). The main problem, then and now, is the fouling of the submerged hulls of ships. Not only does shipfouling cause an increase in the resistance to the passage of ships through water but it has a destructive action upon hull coatings and accelerates corrosion of the hull (9,66).

Marine algae contribute significantly to the fouling communities on a wide range of immersed substrates. However, it is their relatively recent role as the dominant fouling organisms on the hulls of container ships and oil tankers which has commanded attention (42). Early studies showed that ship fouling occurred almost entirely in port. The more intensive and regular the operation of a structure subject to fouling, the fewer the number of species and

individuals per species of fouling organisms observed (9). However, large tankers which spend only a short time in port can be extensively fouled by algae. Also, these vessels often commute between tropical and temperate waters. Generally, only members of the genera Enteromorpha and Ectocarpus foul these vessels. In addition, these algae appear to be largely unaffected by the conventional antifouling compositions used for hull protection (42). Of the two algae, Enteromorpha (the "green" weed) is the more prevalent for the following reasons: it can survive wide extremes of temperature and salinity; it requires only inorganic nutrients and light for growth; it can adhere strongly to surfaces; and, it is not hindered in its growth by the speeds attained by large tankers, but is actually stimulated (39,69). The economic loss resulting from fouling has justified investigations of its cause and, more particularly, of possible methods for its prevention. Using the current generation of high performance anti-fouling techniques, a large tanker (250,000 tons) will lose 0.9 knots or more in speed after 22 months in service. This represents a 2.5% loss in speed equal to \$235,000 on the cost of fuel alone (43). For these tankers the cost for one week in dry dock is \$188,000 to \$282,000 (43,83). These are early 1970 costs and would be considerably higher now. The attachment of algae on the sides of these large tankers can cause 30-37% extra fuel consumption per year (43). The magnitude of these costs has created an increasing demand

for more effective antifouling measures, both with regards to algacidal activity and length of life of the antifouling measures.

During the last four decades, many anti-fouling methods have been proposed and tested. These have varied from isotope introduction into metal or coatings, installations of electrical systems for charge reversal, use of ultrasonics (9), use of chlorine (69), and use of divers for periodic underwater hull cleaning. Still, no effective method of any appreciable longevity has been demonstrated. Toxic paint coatings remain the predominant preventive technique. The current generation of high-performance coatings will provide protection for about one and one-half to two years (79).

Since fouling is a biological phenomenon, it would be useful to have a fundamental understanding of the biotic factors involved in order to develop an effective antifouling system. Presently, the prime focus of the investigation of algal fouling centers on the study of algal growth and development on surfaces. The information gained from these studies should be useful in the development of toxicants which will act more selectively on particular growth stages of the fouling organism.

Enteromorpha clathrata is a green alga (Chlorophyta) and belongs to the order Ulvales. The alga has a tubular thallus which is monostromatic and has a complex system of branching. The alga is attached to a surface by a rhizoidal

outgrowth (holdfast) at the base of the thallus. The rhizoid is formed from a network of cells in long filaments. E. clathrata can reproduce by both asexual and sexual means. Asexual reproduction is accomplished by production of motile zoospores or through regeneration of the whole alga from cells of thallus or rhizoidal origin (19,71). Sexual reproduction is accomplished by production of motile gametes (53,71). Motile gametes or zoospores can attach to surfaces (16,17,34). In a recent study (14), it was shown that individual thallus or rhizoidal cells could also attach to surfaces.

After the initial attachment, the success of an alga as a fouling organism depends upon a number of additional factors. One of the most important considerations is the ability of the alga to form an extensive and efficient anchorage system. Not only is this required to maintain an expanding thallus, but also to combat the effects of wave action and ship movement. Despite the importance of the anchorage system to a fouling alga, very few studies have dealt specifically with this capability (15,42,66). Presently, very little is known about the growth and attachment of rhizoidal filaments of marine fouling algae.

The occurrence of a mucilaginous coating around the rhizoidal filaments of Enteromorpha spp., Ectocarpus fasciculatus, and Ectocarpus siliculosus has been shown (15,42,66). This extracellular material, which is produced at the growing apices of the rhizoidal filaments, probably

serves as an adhesive by facilitating good contact between the rhizoidal filament and the substrate. Although quite effective for the adhesion of outwardly spreading rhizoidal filaments, it is clearly of even greater importance in the adhesion of the thallus structure which is subject to the rigors of speed and wave generated shear. Not only does the rhizoidal system provide anchorage for the erect thallus, it probably also serves as a propagating organ (42). The occurrence of plastids in the rhizoidal filaments of all species investigated indicates that they can survive independently of the thallus cells. Rhizoidal cells are capable of initiating new thallus growth. This ability of the more hardy rhizoidal filaments to assume a vegetative role adds to the chances of survival of the alga, especially during unfavourable periods. It has been shown that even if the erect thalli are killed off by severe environmental changes or scrubbed off mechanically during docking periods, portions of the rhizoidal system that remain can initiate further growth (42).

E. clathrata was the organism chosen for studying the mechanisms of adhesion and the chemical nature of the adhesive material for the following reasons:

- 1) E. clathrata is known to foul the hulls of large tankers (34);
- 2) the ability of E. clathrata to grow by regeneration from a single cell allows for convenient laboratory manipulation (14);
- 3) E. clathrata was found to grow at a faster rate than other species of Enteromorpha tested and

also to form the strongest adhesion to glass (14); 4) E. clathrata can be grown in large enough quantities so that the adhesive material can be isolated and chemically characterized; and 5) I believe that a rational approach to the control of algal fouling requires knowledge of the mechanism of algal attachment and subsequent growth, as well as knowledge of the chemical content of the adhesive material.

The purpose of this study was threefold: 1) to determine the mechanism of adhesion of E. clathrata to glass; 2) to determine the effect of various environmental factors on the adhesion of E. clathrata to glass and, 3) to isolate and characterize the adhesive material with respect to chemical content.

LITERATURE REVIEW

In 1935, Zobell and Allen (97) showed that bacteria were the primary film-formers on submerged glass slides, and that such films favored the subsequent attachment of the larger fouling organisms. They also suggested that bacteria may form a mucilaginous surface to which other fouling organisms might attach. Furthermore, they found that attachment by marine bacteria to a glass surface occurred in two steps: 1) temporary attachment; and 2) firm attachment which took a few hours.

Taylor in 1970 (86), set down the possible mechanisms responsible for the two types of adhesion. He stated that temporary adhesion was due to the attractive forces operating directly between the molecules on the outside of a cell and those of the substratum surface, while firm adhesion resulted from chemical bonds established between the surfaces or between each surface and molecules of a substance which came between them. He also suggested that the production of extracellular material was probably responsible for firm adhesion.

Marshall et al. (59,62,63) confirmed the work of Zobell on the two phases of attachment of marine bacteria. They termed these two phases reversible and irreversible sorption. Reversible sorption was said to be an instantaneous attraction of bacteria to a surface. The bacteria were held weakly near the surface, exhibited

Brownian motion, and were readily removed by washing the surface with 2.5% NaCl. Irreversible sorption was said to involve the firm adhesion of bacteria, loss of exhibition of Brownian motion and lack of removal by washing with 2.5% NaCl.

Marshall et al.(59,62,63) also demonstrated that the initial, reversible sorption of Achromobacter sp. strain R8 to a glass surface could be explained in terms of the Derjaguin-Landau and Verwey-Overbeek (DLVO) theory (63,81). This theory, modified to explain bacterial attachment, states that the total interaction energy of a bacterium to the substrate is determined solely by the sum of the van der Waal attraction energy and the repulsive electrostatic energy. As a bacterium approaches a surface it experiences a weak van der Waal attraction induced by the surface charge on the bacterium and on the surface. This attraction increases as the bacterium moves closer to the surface. However, since both bacterium and surface are negatively charged in an aqueous system a repulsive force comes into effect. As the surfaces approach each other, owing to the overlap of the diffuse layers of counterions associated with each charged surface. The bacteria are captured by a surface in a weak secondary potential energy minimum (attractive trough). Bacteria in the secondary minimum are weakly held to the surface and are prevented from closer approach by the primary electrostatic repulsive forces.

Irreversible adhesion was shown to be a result of the

production of extracellular material (59,62,63). Calcium and magnesium were also required for irreversible adhesion (59,62,63).

In 1973, Marshall and Cruickshanks (61) studied two freshwater bacteria, Flexibacter aurantiacus and Hyphomicrobium vulgare that attached to polystyrene but not to glass. They showed that the initial attachment was a result of hydrophobic attraction. Firm attachment (irreversible attachment) however, still required the synthesis of extracellular adhesive material. This extracellular material was not sensitive to trypsin, but was sensitive to sodium periodate suggesting a carbohydrate material.

Zvyagintsev, et al.(98) observed that after a period of time some microorganisms that were irreversibly attached to a substrate became reversibly attached. They attributed this to the secretion of exoenzymes which disrupted the slime with which the cells were attached to the surface.

Fletcher (38) showed that surface charge density was important in electrostatic interactions between cells and surfaces in some cases, whereas surface-free energy effects (wettability) predominated over electrostatic interactions in other cases. These two factors were probably responsible for differences in attachment to various substrates. She also showed the importance of adsorbed organic compounds to attachment. Both surface charge density and surface free energy can be influenced through adsorption of organic

compounds. Fletcher (36) further showed that attachment of cells to unsuitable surfaces (those not normally adhered to) may be increased through addition of proteins which adsorb on to the substrate. However, attachment to a suitable surface could also be retarded by adsorbed proteins. Several types of adhesion processes have been identified by Marshall and Bitton (60). The one consistent feature of these different types of adhesion was that firm attachment between bacteria and surfaces was established by means of polymer bridging. Permanent adhesion was the phenomenon that referred to the permanent binding of a microorganism to one site on a surface. Various microbial surface components (e.g. capsules, slime layers or the glycocalyx, microfibrils, and fimbriae) have been implicated in the attachment of different organisms to surfaces. Some microorganisms were capable of permanently adhering to many types of surfaces. This ability was referred to as nonspecific adhesion. The extent and strength of nonspecific adhesion by a microorganism to different surfaces may be dependent on the initial surface properties of the solids involved. Specific adhesion of certain microorganisms to particular surfaces must involve interactions between complementary molecular configurations on the surfaces of the solid and the bacteria. Temporary (or Stefan) adhesion is a type of adhesion that allows movement across a surface. This is achieved by the production of a viscous slime that increases adhesiveness

(the force preventing separation) but allows the organism to move across the surface (a relatively low horizontal drag) (60). Stefan adhesion was shown by Humphrey et al.(50) to be the type of adhesion which enabled Flexibacter BH3 to move across a surface.

Fletcher (39) raised the possibility that adhesive polymers undergo a setting process and become permanent adhesives. Setting involves divalent cations which may cross-link the adhesive polymer. Chemical treatment with sodium periodate and disodium tetraborate were shown to disrupt cell adhesion. These two chemicals were known to denature polysaccharides thus suggesting that the adhesive polymer contained carbohydrate (39). Evidence suggesting that the adhesive material was partly proteinaceous was developed through the use of enzymatic digestion with trypsin and pronase (39). In general, the adhesive material of bacteria is believed to consist of both protein and carbohydrate (22,24,29,39,53,59).

The principal fouling organism on large tankers and container ships are algae. Thus, the study of their attachment has become increasingly important. The initial attachment of zoospores of two prominent algal foulers Enteromorpha intestinalis and Ectocarpus spp. has been extensively studied (5,6,12,13,17,34). These investigations showed that when the zoospores settled onto a surface, an extracellular polymer was secreted which was responsible for firm attachment. With the use of α -amylase and trypsin,

Christie, et al.(17) were able to determine that this adhesive polymer was of a protein-carbohydrate nature. Furthermore, they observed a decrease in the ability of these enzymes to affect attachment, suggesting some sort of chemical change or hardening process taking place in the adhesive material.

Callow, Christie and Evans (12,13,34) suggested a possible scheme for the synthesis of the adhesive material in Enteromorpha. The protein moiety is transported to the golgi apparatus from the adjacent rough endoplasmic reticulum. The carbohydrate component of the adhesive material is attached to protein and polymerized in the golgi cisternae after detachment and formation of a cytoplasmic vessicle. Subsequently, the vessicle travels to the cell membrane and is secreted into the area between the zoospores and the substrate.

Algae, like bacteria, have been shown to produce extracellular material which was responsible for firm attachment (15,34,42,66). Cytochemical and enzymatic digestion has shown this algal extracellular material to be composed of a carbohydrate-protein complex (15) analogous to the bacterial extracellular matrix. However, unlike bacteria, algal spores have not been found to be restricted with regard to the types of substrate to which they can attach (15).

Previous investigations into the attachment of E. clathrata (14) showed that the extracellular adhesive

material was composed of protein and carbohydrate. The adhesiveness of this material has been found to be influenced by such environmental factors as light, darkness, temperature, and calcium. An inhibitor of photosynthesis, DCMU, was shown to mimic the dark effect by causing a release in attachment. Various light intensities and wavelengths either increased or decreased adhesion. Temperature was indirectly related to adhesion by affecting growth. Calcium was shown to be specifically required for firm attachment and was thought to cause a hardening or curing of the adhesive material by cross-linking the adhesive polymer chains.

Further investigation into the prevalence of Enteromorpha spp. (approx. 75%) (16) as fouling organisms revealed their adaptability. Christie and Shaw (18) demonstrated the high tolerance of Enteromorpha intestinalis to wide extremes of temperature and salinity. These two factors were thought to provide Enteromorpha with a selective advantage as a tanker fouler, because the tankers were constantly exposed to these environmental extremes. In addition, Enteromorpha spp. were thought to have a further advantage as a colonizing organism because they were able to attach and grow even at the high speeds travelled by these modern ships. When the hull water velocity was in excess of two knots a wide range of sedentary and boring organisms failed to attach (49). In contrast, Enteromorpha spp. were found to attach at hull water velocities up to 10.7 knots

(49). Moreover, the growth of Enteromorpha spp was shown to be stimulated by the speeds attained by these tankers (39,69).

Presently, the method to combat algal fouling is the use of surface paints containing toxic compounds. The majority of antifouling paints have copper as the main toxic compound, but organo-tins are becoming increasingly popular (34). Significantly, Enteromorpha was shown to be highly resistant to these two toxic compounds (16,34,77).

Not only does the growth of Enteromorpha lead to a loss in speed for oil tankers, but it also has a detrimental effect on the paint surfaces. The rhizoid structure of Enteromorpha is composed of long filaments of cells. As these filaments grow, they have been shown to burrow along and under the surface of coating materials (66). This leads to blistering of the surface of the paint leading to corrosion of the exposed metal (9). Thus, control of algal fouling is an important economic concern.

MATERIALS AND METHODS

Organism, Media and Growth

E. clathrata LB1847 #Ea-2 male is a green alga that was obtained from the Indiana Culture Collection, Department of Botany, Indiana University, Bloomington, Indiana and rendered axenic by Dr. Stevens, Jr. (Pennsylvania State University).

Unless otherwise noted, cultures of E. clathrata were grown in 60 ml wide-mouth Florence flasks, on glass slides, or on glass cover slips using an artificial seawater medium designated medium A (see Table 1). All cultures were maintained in an environmental incubator (Controlled Environments Model EY15) set at a temperature of 20°C with illumination provided by ten (overhead) F72T12-CW-1500 fluorescent lights. The continuous incident light intensity was 150 $\mu\text{E}/\text{m}^2/\text{sec}$.

All cultures were inoculated from a stock of homogenized cells prepared by scraping the algae from a stock culture and grinding them in a Ten Broeck tissue homogenizer. The concentration of homogenized cells was adjusted (by adding medium A) so that 1 ml of cells in 20 ml of medium A resulted in an OD_{550} of 0.2 unless otherwise noted. Measurements were made with a Bausch and Lomb Spectronic 20 colorimeter. Either 1 ml of the homogenized cells was inoculated into 60 ml Florence flasks containing

Table 1

Culture Medium A¹

Composition of culture medium:

<u>Compound</u>	<u>g/l</u>
Na ₂ EDTA	0.03
NaCl	18.0
MgSO ₄ ·7H ₂ O	5.0
KCl	0.6
CaCl ₂ ·2H ₂ O	0.37
NaNO ₃	1.0
KH ₂ PO ₄	0.05
Trizma base ²	1.0

Trace element composition:

<u>Compound</u>	<u>mg/l</u>
Vitamin B-12	0.004
FeCl ₃ ·6H ₂ O	3.89
H ₃ BO ₃	34.3
MnCl ₂ ·4H ₂ O	4.3
ZnCl ₂	0.315
MoO ₃ (85%)	0.03
CuSO ₄ ·5H ₂ O	0.003
CoCl ₂ ·6H ₂ O	0.0122

¹Derived from Provasoli et al.(70) modified by Van Baalen(88) and Stevens et al.(85).

²Trizma base was adjusted to pH 8.2 with conc. HCl.

20 ml of medium A or 2.5 ml were inoculated into petri dishes containing glass slides or glass cover slips. All cultures were incubated for 2 weeks unless otherwise noted. A growth period of 2 weeks provided for an even, confluent growth.

The quantitative estimation of growth was made by dry cell weight. The determination of dry cell weight was done by removing the algae from the flasks either by using the method for determining the percentage of adhesion (see below) or by removing all the cells with a rubber policeman. Cells were collected on tared No.1 Whatman filter papers. The cells were then washed several times with distilled water. They were then dried in an oven for 48 hours at 85°C or until a constant weight was obtained. After weighing on an analytical balance, the dry weight of the algal cells was calculated.

Determination of the Percentage of Adhesion

Adhesion of cultures grown in 60 ml flasks was measured using an apparatus modified from Christie, Evans and Shaw (17) and described by Campbell (14). The cultures were hosed with water at a pressure of 12 psi for 15 seconds. The cells that came off the glass were removed and their dry weight was determined as described above. These cells were defined as unattached cells. The cells still attached to the glass were removed with the use of a rubber policeman

and their dry cell weight was determined. These cells were defined as attached cells. The percentage of adhesion (dry weight basis) was then calculated using the following formula:

$$\frac{\text{weight of cells attached}}{\text{total weight of cells}} \times 100 = \% \text{ Adhesion}$$

Adhesion of cultures grown on glass slides was determined by measuring the OD₅₅₀ of 5 different areas of the glass slide and then averaging the resulting values. To accomplish the measurement, the glass slide was placed in a special holder and the OD₅₅₀ was determined with a Cary Spectrophotometer Model 14R. The holder consisted of a plexiglass stand which held the glass slide in an upright position along its length. The height of the stand was fixed so that the light beam from the spectrophotometer passed only through the middle of the slide. The holder was moved 2 cm to the right or left for each OD measurement. The glass slide was then hosed with water at a pressure of 12 psi. The OD₅₅₀ was again determined on the same 5 areas and the values averaged. The percentage of adhesion (OD basis) was then calculated using the following formula:

$$\frac{\text{avg. OD after hosing}}{\text{avg. OD before hosing}} \times 100 = \% \text{ Adhesion}$$

Adhesion of cultures grown on glass cover slips was measured by counting the number of viable algal cells (meaning deep green cells) after rinsing gently five times in medium A. The cover slips were placed in a Sykes-Moore Culture Chamber purchased from Bellco Biological Glassware for counting. The cover slips were either hosed at 12 psi or placed upside down (gravity removal of unattached cells) in the culture chamber filled with an appropriate solution. The number of cells remaining attached were then counted. The percentage of adhesion (cell number basis) was then calculated using the following formula:

$$\frac{\text{\# of cells after treatment}}{\text{\# of initial cells}} \times 100 = \% \text{ Adhesion}$$

Determination of Time of Adhesion

Reversible adhesion. Homogenized cells were washed 3 times in medium A with centrifugation of 3000 rpm for 10 minutes between each wash. Cells were suspended to an OD₅₅₀ of 0.1 in medium A. These cells were inoculated into a Sykes-Moore culture chamber. The chamber was turned upside down at various time intervals, remaining upside down for 30 minutes, after which time, the number of cells attached to the top cover slip (in an area of 100 sq.mm) was counted.

Semi-reversible adhesion. Homogenized cells were washed 3 times in medium A with centrifugation at 3000 rpm for 10

minutes between each wash. Cells were suspended to an OD₅₅₀ of 0.1 in medium A. These cells were then inoculated into 35 mm tissue culture dishes containing round cover slips. At various times, a cover slip was removed, gently rinsed in medium A five times, and placed into a Sykes-Moore culture chamber. The chamber was filled with medium A and turned upside down. Ten minutes later, the cells still attached were counted. The cover slip was then removed and rinsed ten times in distilled H₂O (which has no effect on the viability of the cells). The cover slip was placed back in the chamber which was filled with distilled H₂O. After ten minutes, the cells still attached were counted, and the percentage of adhesion (cell number basis) was determined.

Irreversible adhesion. Homogenized cells were washed 3 times in medium A with centrifugation at 3000 rpm for 10 minutes between each wash. Cells were suspended to an OD₅₅₀ of 0.1 in medium A. The cells were then inoculated into petri dishes containing glass slides. Every 24 hours, a glass slide was removed and gently rinsed in medium A. The OD₅₅₀ was then determined at five different places on the slide. The slide was then washed with water at 12 psi for five seconds. The OD₅₅₀ was again measured and the percentage of adhesion (OD basis) was determined as previously described.

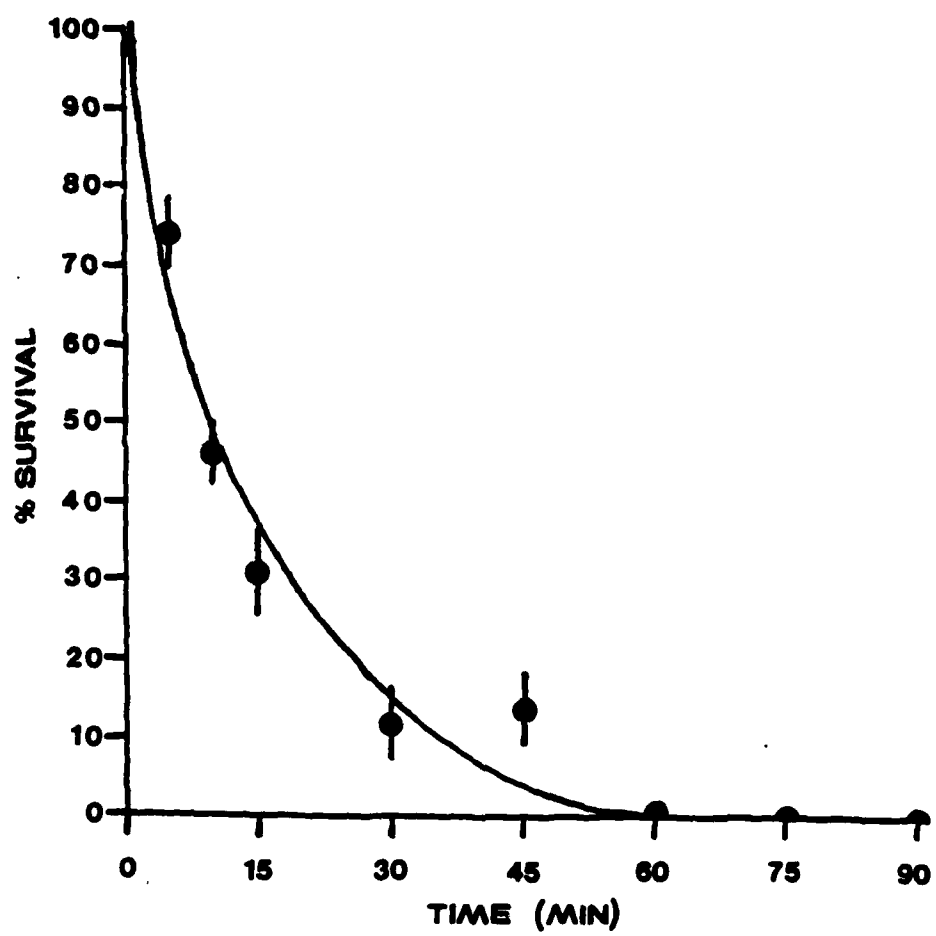
Determination of Active or Passive Processes in Adhesion

In order to determine whether the processes involved in the three types of adhesion were passive or active, cells of E. clathrata were killed by exposure to ultraviolet irradiation. To determine the exposure to UV irradiation necessary to kill cells of E. clathrata, homogenized cells were subjected to various doses of UV irradiation. Homogenized cells were adjusted to an OD₅₅₀ of 0.2 by adding medium A. Subsequently, 5 ml were added to 35 mm tissue culture dishes. The dishes (tops removed) were then placed in a UV irradiation box which contained five G8T5 Sylvania germicidal bulbs. The continuous light intensity was 3.6 J/m²/sec. At various times, the dishes were removed from the UV box with their lids replaced and put into the environmental incubator for 2 weeks. At the end of 2 weeks, the dishes were scraped with a rubber policeman, and the dry cell weight was determined as previously described. Since one hour (Fig.1) was sufficient to kill all the cells of the homogenate, 1.5 hours of UV exposure was used for all experiments.

Reversible adhesion. Homogenized cells were washed three times in medium A with centrifugation of 3000 rpm for 10 minutes between each wash. Cells were suspended to an OD₅₅₀ of 0.1 in medium A. These cells were inoculated into 35 mm tissue culture dishes containing round cover slips. The dishes (tops removed) were then placed in the UV box for 1.5

FIGURE 1

Effect of time of UV exposure on the survival of E.
clathrata. Each point represents the mean of 5 separate
experiments. Error bars indicate \pm standard deviation.



hours. The dishes were removed and placed in the environmental incubator. At various times a cover slip was removed and placed into the Sykes-Moore culture chamber. The chamber was filled with medium A and placed upside down so that those cells not attached would fall away due to gravity. After 30 minutes, the number of cells attached to the cover slip (an area of 100 sq.mm) was counted.

Semi-reversible adhesion. Homogenized cells were washed 3 times in medium A with centrifugation at 3000 rpm for 10 minutes between each wash. Cells were suspended to an OD₅₅₀ of 0.1 in medium A. These cells were then inoculated into 35 mm tissue culture dishes containing round cover slips. Ten hours after inoculation, the dishes (tops removed) were placed in the UV box for 1.5 hours. The dishes were removed and placed into the environmental incubator. Starting at 15 hours post inoculation, a cover slip was removed, gently rinsed in medium A 5 times, and placed into the Sykes-Moore culture chamber. The chamber was filled with medium A and turned upside down for 30 minutes. The cells still attached were counted. The cover slip was then removed and rinsed 10 times in distilled water. The cover slip was placed back into the chamber which was filled with distilled water. After 10 minutes, the cells still attached were counted, and the percentage of adhesion (cell number basis) was determined.

Irreversible adhesion. Homogenized cells were washed 3 times in medium A with centrifugation at 3000 rpm for 10

minutes between each wash. Cells were suspended to an OD₅₅₀ of 0.2 in medium A. These cells were then inoculated into petri dishes containing glass slides. The petri dishes were placed into the environmental incubator for 7 days. Slides were removed each day, and the percentage of adhesion was determined. The pH of the medium in which the slide was suspended was also determined. Other inoculated glass slides were removed from their original petri dishes and placed in new sterile petri dishes. The spent medium was filter sterilized and just enough was returned to the new petri dishes so that the glass slides were barely covered. The medium in some of the petri dishes was adjusted to a pH of 9.5. The petri dishes were placed in the UV box (lids removed) and irradiated for 1.5 hours. The petri dishes were then filled with medium A and placed back into the environmental incubator for 7 additional days. Slides were removed each day, and the percentage of adhesion (OD basis) was determined along with the pH of the medium.

Determination of the Effect of Substratum on Adhesion

Different substrates. Different substrates were trimmed to an area of 1.0 sq.cm and placed in 35 mm tissue culture dishes. These were then inoculated with homogenized cells suspended to an OD₅₅₀ of 0.1. Two weeks later, the substrates were removed and counts made through a stereomicroscope. The substrates were then washed with

water at a pressure of 12 psi. Counts of E. clathrata were again made, and the percentage of adhesion (cell number basis) was determined as previously described.

Adsorbed polymers. Homogenized cells were washed 3 times in distilled H₂O with centrifugation at 3000 rpm for 10 minutes between each wash. Cells were suspended to an OD₅₅₀ of 0.2 in medium A. The polymers selected were proteins covering a wide range of isoelectric points and are as follows:

Pepsin, 1:60,000	pI 1.0
Bovine Serum Albumin, fraction V	pI 4.7, 4.9
Gelatin, type I	pI 4.7, 5.0
Cytochrome C	pI 10.65
Histone, type II	pI 10.8
Protamine Sulfate, grade I	pI 12.4

The proteins were adsorbed onto glass slides by placing the slides in a 0.1% solution of the protein (see below). The slides remained in the protein solution for 3 hours. They were then carefully rinsed in distilled water 3 times. Next, the slides were placed in petri dishes, and the algal suspension was added. Cultures were incubated for two weeks, and the percentage of adhesion (OD basis) was determined as previously described.

The slides that were coated with proteins developed an obvious change in their wettability which provided a qualitative indicator of protein adsorption. To further

test for adsorbed proteins on the slides, they were immersed in a 1:4 solution of Bio-rad protein-dye binding reagent for 45 minutes. After this time the slides were removed, rinsed in distilled water and placed in the slide holder for analysis at 595 nm. Slides with adsorbed proteins on their surface had an OD_{595} of 0.1-0.15, while control slides had an OD_{595} of 0.02-0.05. The coating of glass slides in a 1% protein solution resulted in an OD_{595} of 0.1-0.15 similar to the 0.1% protein solution. Thus, the latter concentration was used. Proteins were obtained from Sigma Chemical Company. The protein-dye binding reagent was supplied by Bio-Rad Laboratories, Richmond, California.

Determination of Electrophoretic Mobility

The determination of electrophoretic mobility was accomplished by using a modification of the procedure of Marshall (58) employed in the laboratory of Dr. Paul Todd, Department of Biochemistry, Microbiology, Molecular and Cell Biology, Pennsylvania State University. All measurements were made at 25°C in a Zeiss-Cam-Apparatus Microelectrophoresis System with Ag/AgCl/KCl electrodes. The following buffers (I=0.015) were used in determining the mobilities at different values of pH:

pH 2-3 Glycine hydrochloride-glycine

pH 4-5 Acetic acid-sodium acetate

pH 6-8 Potassium dihydrogen phosphate-disodium
hydrogen phosphate

pH 9-11 Sodium bicarbonate-sodium carbonate

Homogenized cells were washed in distilled H_2O and centrifuged at 3000 rpm for 10 minutes. These homogenized cells were added to 50 ml of the appropriate buffer until the OD_{550} reached 1.0. At least 39 different cells in each buffer were timed in both directions. The electrophoretic mobility and standard deviations were obtained with a computer program originally written in Fortran by R.A. Gaines and converted to Basic by L.D. Plank for use on the Departmental PDP 11/10 computer.

Determination of the Effect of Electrolyte Concentration on Adhesion

Reversible adhesion. Homogenized cells were washed 3 times in distilled H_2O and then 3 times in the appropriate electrolyte solution, with centrifugation at 3000 rpm for 10 minutes following each wash. The cells were added to 20 ml of the electrolyte solution until an OD_{550} of 0.1 was reached. The electrolyte solution consisted of various concentrations of $NaCl$, $CaCl_2$ or $MgSO_4$. The cells were then inoculated into a Sykes-Moore culture chamber and immediately turned upside down (viewing side was now up). A 30 minute period was allowed before counting to allow those

cells not attached at the liquid-glass interface to fall from view by gravity. A 1.0 sq.cm area of those cells that remained attached was counted. This experiment was repeated except that polystyrene cover slips were used instead of the usual glass cover slips. The percentage of adhesion (cell number basis) was determined as previously described. The theoretical thickness of the diffuse double layer ($1/K$) was calculated from the expression

$$K=0.327 \times 10^8 \ z \sqrt{c}$$

where z =valency and c =molar concentration of electrolyte (63). This expression holds for aqueous solutions of symmetrical electrolytes at 25°C (81).

Semi-reversible adhesion. Homogenized cells were washed 3 times in distilled H₂O with centrifugation at 3000 rpm for 10 minutes between each wash. Cells were diluted to an OD₅₅₀ of 0.1 and inoculated into 35 mm tissue culture dishes containing round glass cover slips. Forty-eight hours later the cover slips were removed, carefully rinsed in 2.5% NaCl five times, then placed in the Sykes-Moore culture chamber. The chamber was filled with medium A and turned upside down. Thirty minutes later, the attached cells were counted. The cover slips were removed and rinsed in distilled H₂O 5 times and in the appropriate electrolyte solutions 3 times. The cover slips were placed back into the chamber. The chamber was then filled with the electrolyte under study and turned

upside down. Thirty minutes later, the number of attached cells were counted and the percentage of adhesion (cell number basis) was measured as previously described.

Irreversible adhesion. Two week old cultures grown in 60 ml wide mouth flasks were rinsed 5 times with distilled H₂O and 3 times with the appropriate electrolyte solution. Twenty ml of the electrolytes NaCl, MgSO₄ or CaCl₂ at various concentrations were added for 30 minutes. The percentage of adhesion (dry weight basis) was then determined as previously described.

Determination of Hydrophobicity

Homogenized cells were washed 3 times in distilled H₂O, with centrifugation at 3000 rpm for 10 minutes following each wash. The cells were added to 20 ml of distilled H₂O to an OD₅₅₀ of 0.2. The cells were then inoculated into a Sykes-Moore culture chamber which had been half filled with immersion oil. The chamber was viewed after 30 minutes of incubation to see if the cells aligned themselves at the oil-water interface.

Determination of the Effect of pH on Adhesion

Reversible adhesion. Homogenized cells were washed 3 times in distilled H₂O with centrifugation at 3000 rpm for 10

minutes after each wash. The cells were resuspended in the appropriate buffer ranging from pH 2 to pH 11. The buffers were the same as those used in determining electrophoretic mobility. The concentration of cells was adjusted to give an OD₅₅₀ of 0.1 in 10 ml of solution. The cells were placed in a Sykes-Moore culture chamber which was turned upside down so that any cells not attached would fall away. Cells were allowed to settle for 30 minutes and then a 100 sq.mm area was counted. The percentage of adhesion (cell number basis) was measured as previously described.

Semi-reversible adhesion. Homogenized cells were prepared as described above for reversible adhesion. The cells were inoculated into 35 mm tissue culture dishes containing round cover slips and incubated for 4 days at 20°C. At the end of 4 days, the cover slips were removed one at a time and placed in a Sykes-Moore culture chamber where the number of cells attached were counted. The appropriate buffer was added. The chamber was turned upside down and shaken gently on an oscillatory shaker for 15 minutes. The buffers used were the same as those used in determining electrophoretic mobility. The percentage of adhesion (cell number basis) was determined as previously described.

Irreversible adhesion. Cultures were grown in 60 ml wide-mouth flasks for two weeks. The media was then removed and replaced with 30 ml of the appropriate buffer. The buffers used were the same ones used for determining electrophoretic mobility. The cultures were shaken on an oscillatory shaker

for 15 minutes. The percentage of adhesion (dry cell weight basis) was then determined as previously described.

Determination of the Effect of Darkness and DCMU on the pH of the Medium

To study the effect of prolonged darkness on the pH of the medium, 2 week old cultures grown in 60 ml wide mouth flasks were wrapped in aluminium foil. To study the effect of DCMU on the pH of the medium, DCMU was added to the cultures at a concentration of 10^{-5} M. The cultures were incubated in an environmental incubator. The pH and percentage of adhesion (dry cell weight basis) were determined daily over a period of 4 days. DCMU was obtained commercially from Agway as Karmex Diuron, and then purified.

Determination of the Effect of Darkness at Constant External pH

To study the effect of prolonged darkness on the adhesion of E. clathrata at constant pH, 2 week old cultures were wrapped in aluminium foil. A continuous flow of medium A (pH 9.5) was furnished to the cultures so that the pH remained at 9.5. The flow rate was set at 100 ml/hr. The pH of the effluent was checked periodically. At the end of 4 days, the percentage of adhesion (dry cell weight basis)

was determined as previously described.

Determination of the Effect of Growth on External pH

Homogenized cells were inoculated into 60 ml flasks and incubated in medium A as previously described. Both pH and percentage of adhesion (dry cell weight basis) were determined every 48 hours. Another set of flasks was inoculated to study the effect of growth on adhesion when the pH was maintained at 7.7. A continuous flow of medium A (pH 7.7) was furnished to the cultures so that the pH remained at 7.7. The flow rate was set at 100 ml/hr. The pH of the effluent was checked daily along with percent adhesion.

Determination of the Role of Calcium in Adhesion

Spent medium was removed from 2 week old cultures which were then washed once with distilled water. Medium A minus calcium was added back to the cultures, which were then placed back into the environmental incubator. At the end of 4 days the medium was removed. Medium A plus 1 mCi calcium-45 was then added to the cultures, which were returned to the incubator. Four days later, the medium was removed, and the cultures were washed 5 times with medium A. Medium A was added to the cultures which were then subjected

to various experimental procedures. Samples of 100 μ l were added to scintillation vials along with 3 ml of scintillation fluid. The radioactivity was determined on a Beckman LS-7000 Liquid Scintillation Counter. To determine the amount of calcium-45 incorporated into the cells, they were washed 5 times with distilled water followed by the addition of 30 ml of concentrated sulfuric acid and 0.1 ml of concentrated nitric acid. The flasks were kept at a near boil on a hot plate for 7 days. Each day, 0.1 ml of nitric acid was added. At the end of 7 days, 1 ml of the mixture was removed from the flasks and neutralized with 1 ml of 17 M NaOH. A 100 μ l sample was added to a scintillation vial along with 3 ml of scintillation fluid. The radioactivity was then determined.

Calcium-45 (S.A.=16.37 mCi/mg) was obtained from New England Nuclear, Boston, Massachusetts.

Determination of the Effect of Enzymes on Adhesion

Homogenized cells were inoculated into 60 ml flasks and incubated as previously described. The enzymes trypsin, protease, and α -amylase were used at a concentration of 1.0 mg/ml which was a sufficient concentration of enzyme to maximize hydrolysis of the adhesive material (14). Each experiment consisted of three sets of 16 flasks. Each set was incubated with one of the three enzymes at various times during growth to determine their effect on adhesion during

the different phases of attachment. In other words, on day 1, one flask from one of the sets had one of the enzymes added. The enzyme remained in the flask till the end of the growth run (sixteenth day). Enzymatic activity was retained during the course of the experiment (see below). On day 2, another flask from the same set had the same enzyme added which remained in the flask till the end of the growth run. This procedure was continued for each flask in a set and with each enzyme. On the sixteenth day of the growth run the percentage of adhesion (dry cell weight basis) was determined on all cultures.

To test the effectiveness of α -amylase in medium A (pH 9.5) at 20°C the assay of Varner and Mense (89) was used. This consisted of adding 100 mg of enzyme to 100 ml of medium A containing 0.15 g of starch. The test solution was incubated for one minute at 20°C. At the end of 1 minute, iodine was added, and the absorbance at 620 nm was measured. After 1 minute, α -amylase gave a positive result. This was characterized by a negative iodine reaction.

To test the effectiveness of trypsin and protease in medium A (pH 9.5) at 20°C, the proteolytic test substrate Azocoll was used. Azocoll is a collagen matrix with an azo dye incorporated into it. As the matrix is digested by an enzyme, the dye is released. The release of dye over a period of time is a measure of the effectiveness of the proteolytic enzyme under study. The 2 enzymes used were separately dissolved in medium A (pH 9.5) at a concentration

of 1.0 mg/ml. Fifty mg of Azocoll were added to 5 ml of enzyme solution. The absorbance was read at 520 nm. Both enzymes completely digested Azocoll in 2 hours. The above tests were performed each day on cultures that had enzymes added, to test for their continued effectiveness. However, every third day fresh enzyme solutions were added.

Azocoll was purchased from Calbiochem-Behring Corporation. The 3 enzymes were obtained from Sigma Chemical Company.

Determination of the Effect of Chemical Treatment on Adhesion

Two week old cultures in 60 ml wide mouth flasks were rinsed with distilled water three times. The cultures were then treated for 30 minutes with sodium periodate, disodium tetraborate, or sodium pyrophosphate. These compounds were dissolved in medium A at a concentration of 0.02 M. The pH was adjusted to 9.5. After treatment, the percentage of adhesion (dry cell weight basis) was determined.

The 3 chemicals were obtained from Sigma Chemical Company.

Determination of Chemical Composition of the Adhesive Material

E. clathrata was grown in 80 large low form flasks under standard conditions in the environmental chamber for 2 weeks. At the end of 2 weeks, the medium was replaced with medium A minus calcium. Four days later the algae were removed with the aid of a rubber policeman. The flasks were rinsed with distilled water and further scraping was continued until no detectable cell material was on the glass surface of the flasks. The adhesive material was extracted from the glass surface by the phenol extraction method of Westphal and Jann (92).

Protein concentration was measured by the protein-dye binding reagent technique of Bradford (11) as supplied by Bio-Rad Laboratories (Richmond, Ca.).

Carbohydrate concentration was measured by the phenol H_2SO_4 technique of Dubois et al. (33).

Uronic acid was determined by the colorimetric carbazole method of Dische as modified by Kabot and Mayer (52).

To elucidate carbohydrate content of the adhesive material, samples containing approximately 100 ug were methanolized and derivatized according to a modification of the procedure of Rickert and Sweely (74). Samples of approximately 100 μg were placed in a glass ampoule. One ml of 0.5 N methanolic HCl was added and a steady stream of

argon was passed over the mixture. The ampoule was then sealed and placed in an oven. The methanolysis was carried out at 100°C for 24 hours. The ampoule was cooled and the seal was broken. The acid was neutralized by placing the ampoule in a closed container containing KOH and CaCl₂ (anhydrous). When the samples were completely dry (approximately 72 hours), the sample was removed by dissolving in 3 ml methanol. Re-N-acetylation was performed by adding 10 mg of silver carbonate followed immediately by 0.1 ml of acetic anhydride. Samples were mixed and allowed to react at room temperature for a minimum of 6 hours. Methanolic supernatants were removed and dried with argon prior to TMSi derivatization. The silylating mixture (100 ul of Hexamethyldisilazane trimethylchlorosilane pyridine, 3:1:9) was added and the reaction vessel sealed. The contents were carefully mixed and left at ambient temperature for 30 minutes. A syringe with a long needle for on-column injection was filled with 5 ul of the sample and injected into the gas chromatograph. Methanolic HCl and the silylating reagent were supplied by Supelco, Bellefonte, Pa. A Perkin-Elmer Sigma 3B gas chromatograph, equipped with a hydrogen flame ionization detector, was used for all analyses. The gas chromatograph was operated with a stainless steel column (6 ft x 1/8 in I.D.) which had been packed with 3% SP-2100 silicone liquid phase on Supelcoport (100-200 mesh) (Supelco, Bellefonte, Pa.). The trimethylsilyl derivatives were assayed by continuous

temperature programming from 140 to 240°C at 0.5°/min with nitrogen as the carrier gas.

Peak areas were measured along with the retentions relative to the internal standard mannitol and compared to results of known sugars.

Pure cell wall from E. clathrata was prepared according to the procedure of Dodson and Aronson (32). Cultures in 20 large low form flasks were extensively washed with distilled water and then removed. The algae were quickly frozen in liquid nitrogen and then ground with a mortar and pestle. The ground algae were mixed with 100 ml of distilled water and homogenized for 1 minute in a Sorval Omnimixer. During this and all subsequent steps in the isolation procedure the material was maintained in cold (3-5°C) 0.1 M Tris/HCl buffer (pH 7.5). One minute of homogenization followed by a short cooling period was repeated 5 times. The fragmented algae were then concentrated by centrifugation at 3000 rpm. The concentrated material was resuspended in 20 ml of buffer. Twenty ml of glass beads (150 mesh, Superbrite Glass Beads) were added, and the suspension was sonicated in a Sonifier Cell Disruptor Model W185 for 30 minutes. Six treatments totalling 3 hours were required for maximum breakage of the cells. After sonication, wall material was separated from glass beads by decantation. The wall fractions were freed of cytoplasm by repeated washings in distilled water followed by centrifugation at 3000 rpm. This was continued until I₂KI stained walls showed no

detectable cytoplasmic contamination. The purified cell walls were freeze-dried giving a gray-white material which was used for all chemical analysis. Phenol extraction and chemical analysis were performed in the same manner as for the adhesive material.

To determine if the adhesive material was part of the cell wall, samples were separated by descending paper chromatography (Whatman No.1, 5X20 in) with a solvent system composed of n-butanol pyridine water (6:4:3) (10). The solvent was allowed to run off the bottom of the chromatogram. The Whatman paper was allowed to equilibrate for 24 hours before sample development. Development of the chromatogram required 48 hours. The chromatogram was stained with either ammoniacal AgNO_3 or bromophenol blue (10).

Electron Microscopy

Cells of E. clathrata were grown on hardened Spurr low-viscosity resin (trimmed to fit inside an embedding capsule) for 2 weeks under standard conditions. Cells were prepared for thin sectioning by the ruthenium red procedure of Balkwill (7) and embedded in Spurr low-viscosity resin. Sections were cut on an LKB Ultratome III ultramicrotome and retrieved with uncoated, 400 mesh, copper grids. After poststaining the thin sections 15 minutes in 3% uranyl acetate (in 50% methanol) and 10 minutes in 0.4% lead

citrate (73), they were viewed at 60kV with a Philips EM 300 electron microscope. Areas of interest were photographed on Kodak Electron Microscope Film (Type 4489), using a 20 um objective aperture.

RESULTS

Periodically bacterial contamination was observed during the course of an experiment, however, when the experiment was repeated with an axenic culture there was no difference in the experimental outcome. Cultures that became contaminated were purified by grinding them up in a Ten Broeck tissue homogenizer and reculturing a single cell.

Time Course for Onset of Adhesion

Reversible adhesion. Cells were introduced into a Sykes-Moore chamber (right side up) at zero time. At various times thereafter the chamber was turned upside-down. As shown in Figure 2, at 1 hour, 92% of the cells fell away from the glass surface due to gravity. By 4 hours only 50% of the cells fell away due to gravity. The maximum number of cells were reversibly attached to glass by 6 hours. Reversible adhesion was characterized by cells attached to glass that would not fall away due to gravity in medium A or in 2.5% NaCl solution, but would detach in distilled water. Rinsing the glass surface with medium A or 2.5% NaCl solution resulted in removal of the cells from the surface of the glass. Brownian motion was also exhibited.

Semi-reversible adhesion. The time required for cells of E. clathrata to attain semi-reversible adhesion is shown in Figure 3. Semi-reversible adhesion was characterized by

FIGURE 2

Time required for the maximum number of cells of E. clathrata to become reversibly attached to glass. Each point represents the mean of 10 experiments. Error bars indicate \pm standard deviation.

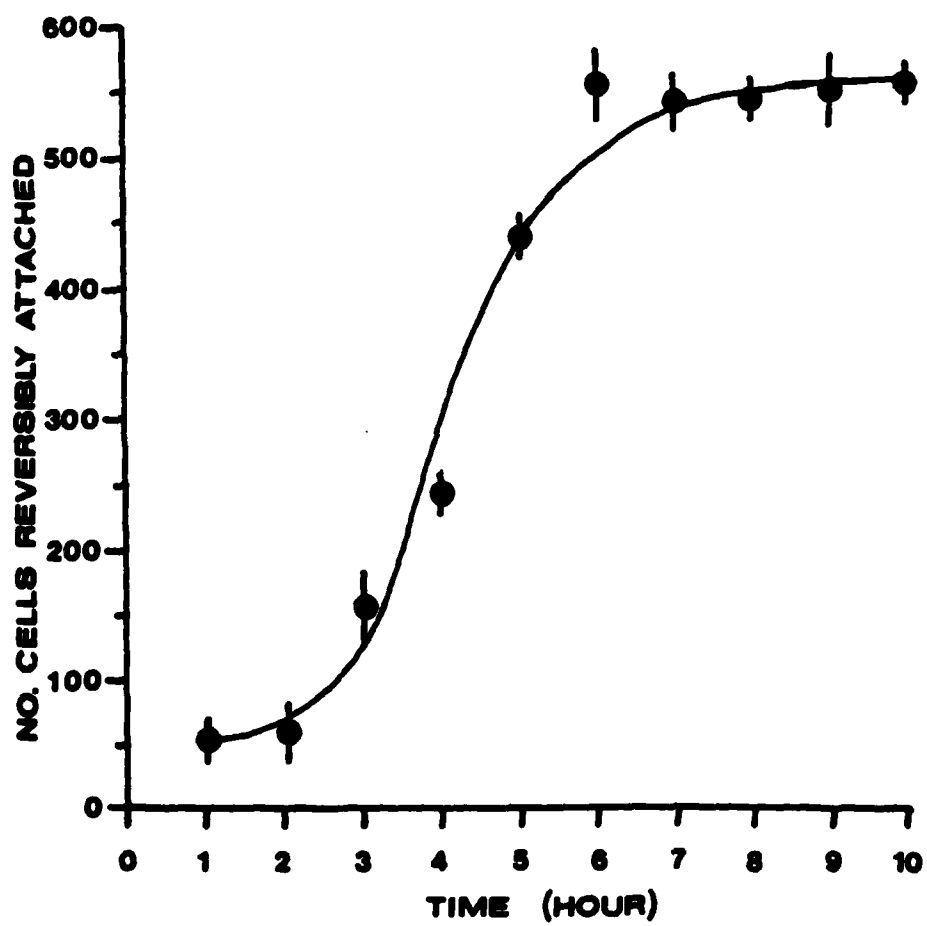
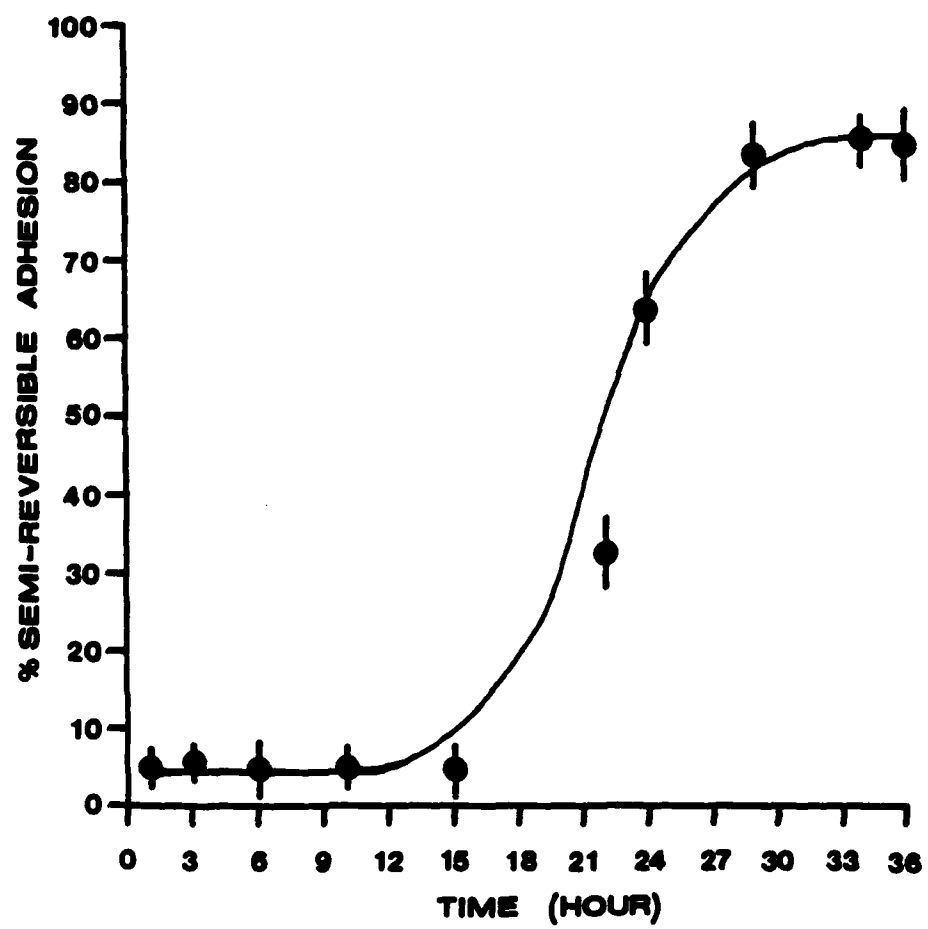


FIGURE 3

Time required for cells of E. clathrata to become semi-reversibly attached to glass. Each point represents the mean of 10 experiments. Error bars indicate \pm standard deviation.



lack of Brownian motion by cells and by the fact that the cells were not rinsed from the glass surface with distilled H₂O. However, cells were removed from the glass by a stream of water under low pressure. Cells began to exhibit semi-reversible adhesion after approximately 12 hours of incubation. By 22 hours, 50% of the cells exhibited semi-reversible adhesion. Maximal semi-reversible adhesion was attained by 30 hours.

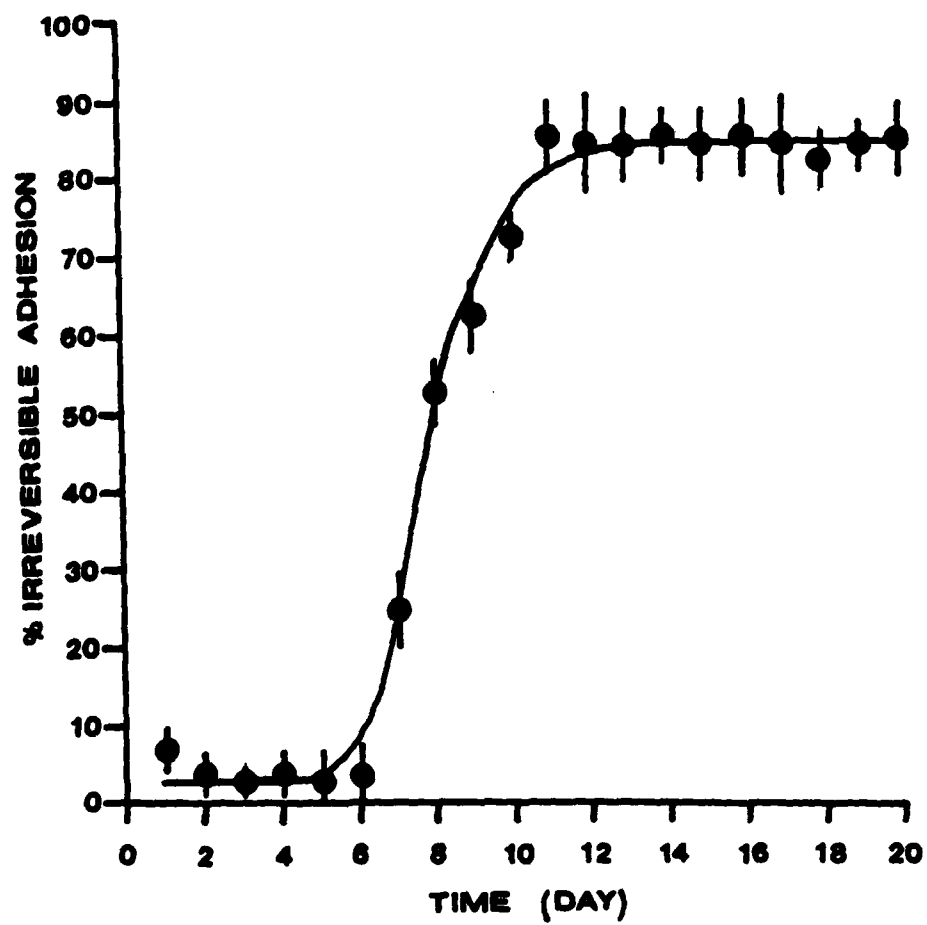
Irreversible adhesion. The time required for cells of E. clathrata to become irreversible attached to glass is shown in Figure 4. Irreversible adhesion was characterized by cells so strongly attached to the surface of glass that hosing with water at 12 psi would not remove them. Cells began to exhibit irreversible adhesion after 7 days of incubation. By the eighth day of incubation 50% of the cells were irreversibly attached. The maximum level of irreversible adhesion (about 90%) was exhibited within 11 days.

Time course of adhesion demonstrated with electron microscopy.

Tissue culture dishes containing pieces of cured Spurr's resin held in place with Ion Agar (to prevent floating) were inoculated with cells of E. clathrata. At various times a piece of Spurr's resin was removed from the growth medium and prepared for observation with an electron

FIGURE 4

Time required for cells of E. clathrata to become irreversibly attached to glass. Each point represents the mean of 10 experiments. Error bars indicate \pm standard deviation.



microscope. The samples were stained with ruthenium red which has been used to specifically stain acidic polysaccharide (40). Good preservation of morphology and contrast of structures (Fig. 5A-D) resulted from the ruthenium red fixation. The alga cell wall was composed of an outer and inner layer. The inner layer was multi-laminate and fibrous while the outer layer was very electron dense and lacked apparent internal structures (Fig. 5A). As previously shown (Fig. 2), by sixty minutes post inoculation the algal cells were reversibly attached. Blebs of the outer cell wall were present at 60 minutes; however, no noteworthy interaction between outer cell wall material and the resin substrate was apparent. By 35 hours (Fig. 5B) a fibrous, reticular substance which stretched between the algal cell and the substrate was observed. This material was also stretched between and around adjacent cells. The fibrous extracellular material was only noticable when the cells had entered into semi-reversible adhesion (see Fig. 3). The material was then present throughout the remaining 14 day growth period (Fig. 5C-D). On day 14 the algae were irreversibly attached (see Fig. 4) and the extracellular material appeared to be very dense and compact (Fig. 5D).

Effect of UV Irradiation on the Stages of Adhesion

Ultra-violet irradiation was used to kill cells of E. clathrata in order to test whether or not dead cells were

FIGURE 5

Thin sections of cells of E. clathrata undergoing the three phases of attachment.

A. Sixty minutes post inoculation. Typical cellular morphology showing an inner (I) and outer (O) layer of the cell wall. There were some blebs present (Bl) which were associated exclusively with the outer layer of the cell wall. The cell is not attached to the substrate (S). An artifact (Ar) believed to be a sliver of resin was present. This picture was representative of 10 separate viewings. Ruthenium red fixation. Bar marker represents 1.0 um. B. Thirty-five hours post inoculation. A large amount of extracellular material (Ex) was present which bridged the gap between the cell surface (CS) and substrate (S). This picture was representative of 15 separate viewings. Ruthenium red fixation. Bar marker represents 1.0 um. C. Four days post inoculation was identical to B. This picture was representative of 15 separate viewings. Ruthenium red fixation. Bar marker represents 1.0 um. D. Fourteen days post inoculation. The extracellular material (Ex) has become more dense and compact. This picture was representative of 15 separate viewings. Ruthenium red fixation. Bar marker represents 1.0 um.



capable of the various stages of adhesion. Cells of E. clathrata that were killed by exposure to UV irradiation did not show any obvious physical change for a period of 8-9 days. The only noticable change was a slight loss of chlorophyll. By the criterion of light microscopy the integrity of the cell was maintained. The only consequence of a cell undergoing passage from one stage of adhesion to another lies in whether it was an active (dependent on the living cell) or passive (dependent on physiochemical reaction/s) process.

Reversible adhesion. The time course of reversible adhesion exhibited by UV killed cells is shown in Figure 6. It should be noted that the curve shown in Figure 6 is virtually indistinguishable from the curve for the reversible adhesion of live cells shown in Figure 2.

Semi-reversible adhesion. The time course of semi-reversible adhesion by UV killed cells of E. clathrata is shown in Figure 7. Over a period of 65 hours only 8-9% of the cells were observed to fulfill the criteria for semi-reversible adhesion. In contrast, living cells were semi-reversibly attached at maximal levels within 30 hours.

Irreversible adhesion. The time course of irreversible adhesion by UV killed cells of E. clathrata is shown in Figure 8. Cells were exposed to UV irradiation on day 7 at which time the pH of the culture was 8.5. In the UV killed culture no further change in the pH was observed. After exposure to UV irradiation an increase in irreversible

FIGURE 6

Time course for reversible attachment to glass of UV killed cells of E. clathrata. Cells were killed with UV irradiation at 0 hours. Each point represents the mean of 5 separate experiments. Error bars indicate \pm standard deviation.

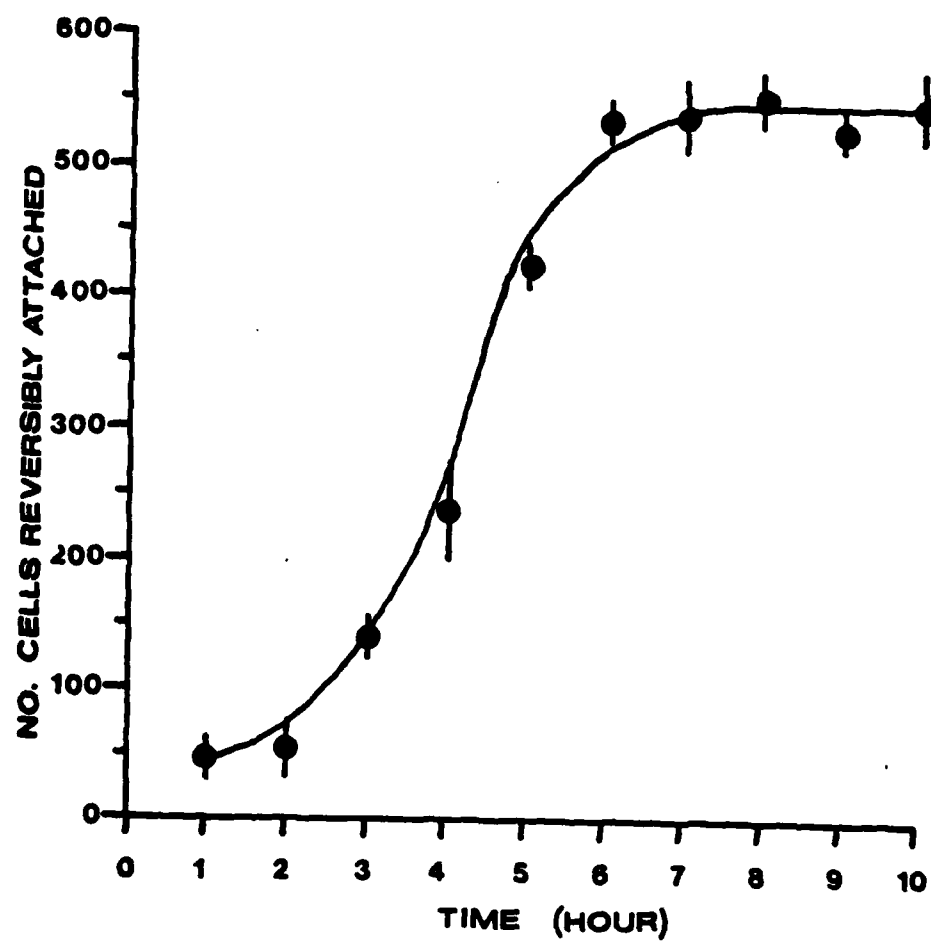


FIGURE 7

Time course for semi-reversible attachment to glass of UV killed cells of E. clathrata. Cells were killed with UV irradiation at 10 hours (indicated by arrow). Each point represents the mean of 5 separate experiments. Error bars indicate \pm standard deviation.

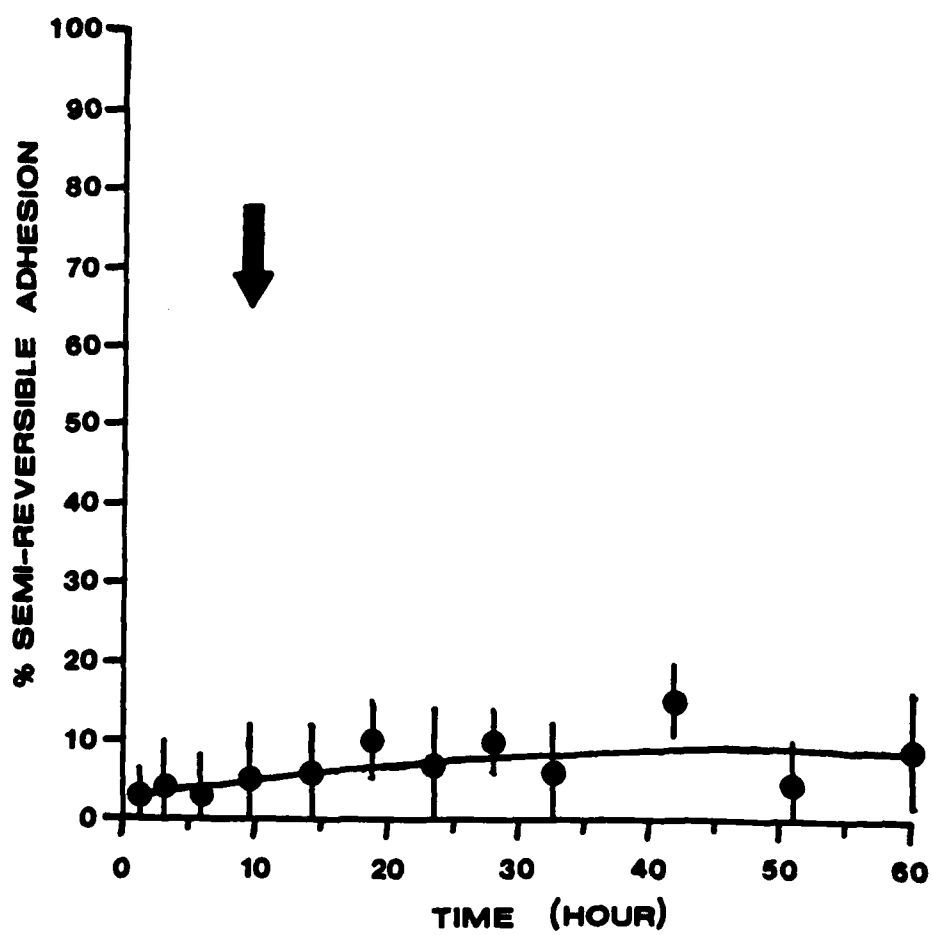
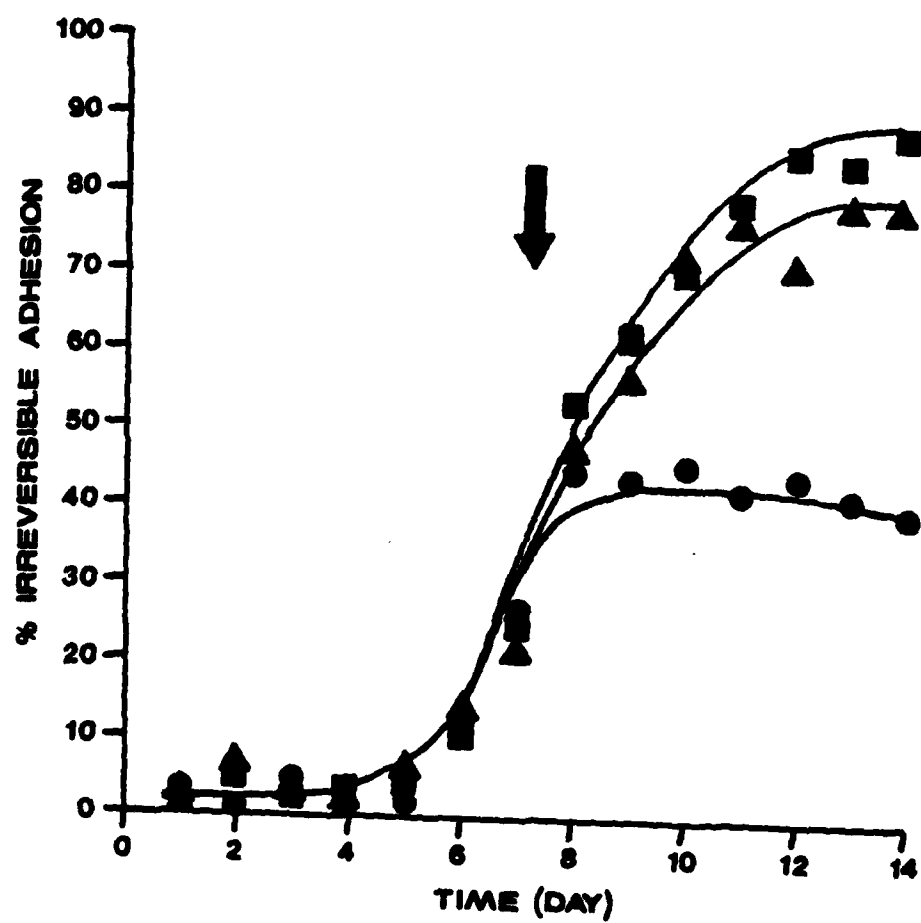


FIGURE 8

Time course for UV killed cells of E. clathrata to become irreversibly attached to glass. Irreversible adhesion of unkilld cells, pH 9.6, ●-●; Irreversible adhesion of UV killed cells, pH 8.5, ■-■; Irreversible adhesion of UV killed cells, pH 9.5, ▲-▲. Arrow indicates time of exposure of cells to UV irradiation. The standard deviation was $\pm 9\%$ for 5 separate experiments.



adhesion of about 12% was observed before the curve flattened out. However, if the pH of a UV killed culture was adjusted to pH 9.5 with NaOH, irreversible adhesion continued to increase with time to a value near 80%. This represents a doubling in the amount of irreversible adhesion when compared to the UV killed culture in which the pH was only 8.5. Moreover, the pH 9.5 UV killed culture was almost as competent as the unkilld culture for irreversible adhesion attaining nearly 80% irreversible adhesion as compared to 90% for the unkilld culture.

Effect of Substratum on Adhesion

Different substrates. The physiochemical properties of various substrata can differ enormously and bacterial attachment to these surfaces vary accordingly. A number of in situ studies have been carried out by submerging different materials in the sea, retrieving these samples at various time intervals, and evaluating the organisms which have attached (31,80). In most cases, with different materials different types and numbers of attached microorganisms were observed, especially during the first few days of submersion (80). In vitro studies have indicated a preference by different bacteria for attachment to different substrates (31,37,41). However, E. clathrata attached to a wide range of substrates as shown in Table 2. There appeared to be no specificity in the type of substrate

Table 2

The Effect of Different Substrates on Attachment¹

Substrate	No. Cells	No. Cells	%
	before wash	after wash	Adhesion
Aluminum	596	564	94
Wood	584	555	95
Glass	610	549	90
Polystyrene (tissue)	601	547	91
Polystyrene (bacteriological)	576	513	89
Resin ²	584	526	90

¹Based on a mean of 5 different experiments with an average standard deviation of 20.

²Resin=Spurr's low viscosity embedding resin.

to which E. clathrata would attach. Polystyrene is representative of hydrophobic surfaces with a critical surface tension for a petri dish of 33 dyne/cm and for a tissue culture dish of 56 dyne/cm (55). The resin substrate (Spurr's low viscosity embedding resin) also has a hydrophobic surface (critical surface tension unknown). Glass is representative of hydrophilic surfaces and has a critical surface tension of greater than 74 dyne/cm (2). Wood represents an organic surface with very low surface free energy and high polarity, hence excellent wettability (80). Aluminum which represents a negatively charged metal surface (80) also provided a good substrate for the adhesion of E. clathrata.

Adsorbed polymers to a substrate. Polymers adsorbed onto substrates have been shown to inhibit subsequent attachment of bacteria. The proteins bovine serum albumin, gelatin, fibrinogen, and pepsin inhibited the attachment of a Pseudomonas sp. to polystyrene (36). Conversely, many adsorbed inorganic and organic compounds facilitated the subsequent attachment of bacteria to a substrate (36). However, an assortment of proteins with a wide range of isoelectric points had no effect on the attachment of E. clathrata to glass or polystyrene (Table 3). The wide range of isoelectric points provided a varied assortment of charge groups on the surface. Not surprisingly, even the diverse population of inorganic and organic substances found in

Table 3

Effect of Adsorbed Polymers on Adhesion¹

Treatment	% Adhesion	% Adhesion
	on Glass	on polystyrene
Control	85	89
Bovine Serum Albumin	95	92
Gelatin	89	91
Protamine Sulfate	96	88
Pepsin	86	92
Histone	85	89
Cytochrome	85	87
Spent Medium A	89	91

¹Based on a mean of 5 separate experiments with an average standard deviation of ± 5 .

spent medium A had no effect on the attachment of E. clathrata (Table 3).

Electrophoretic Mobility

The electrophoretic mobility of single cells of E. clathrata at various values of pH is shown in Figure 9. The results indicated that the mobility of E. clathrata was relatively constant and that the differences in mobility values from -1.4 to -1.9 are comparatively small (58). The large standard deviation encountered was due to the large cell size and the nonuniformity in cell size. Both of these factors have a large effect on the frictional co-efficient (1).

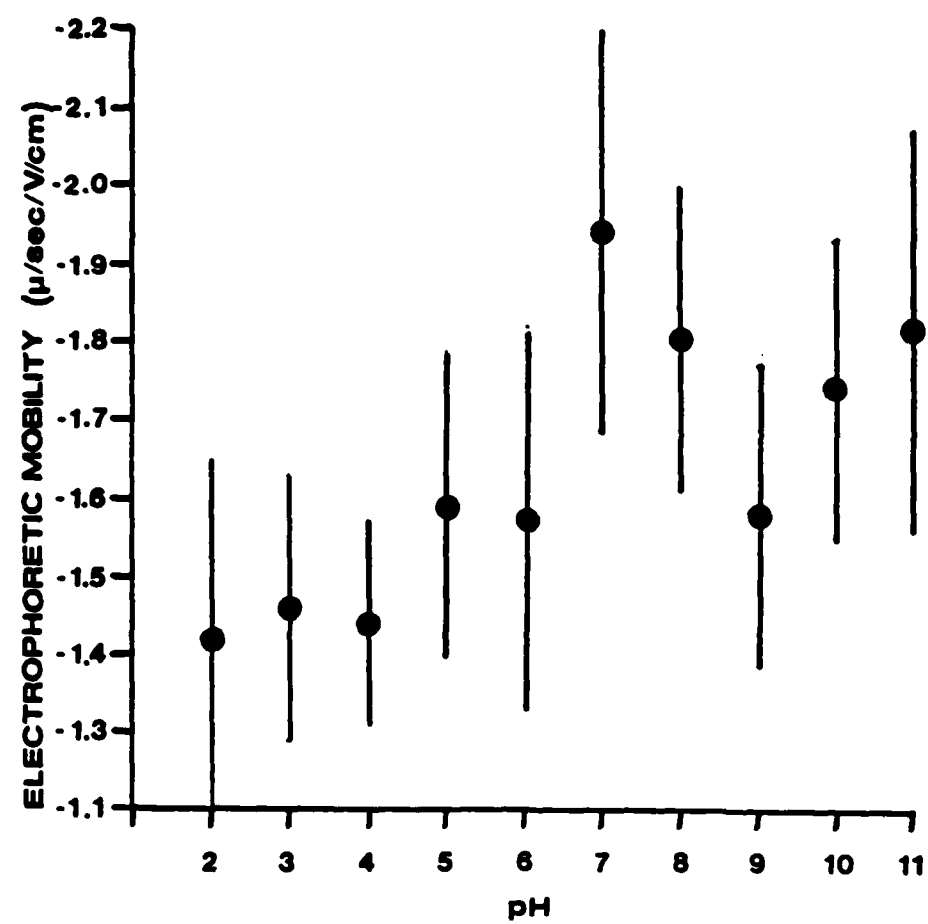
The electrophoretic mobility of a cell can be used to determine the net surface charge on that cell. A negative mobility indicates a net negative charge on a cell surface while a positive mobility indicates a net positive charge. Thus, the negative mobility of E. clathrata indicated that the cell surface had a relatively stable net negative charge over a wide range of pH values.

Effect of Electrolyte Concentration on Adhesion

Reversible adhesion. Reversible adhesion of E. clathrata was shown to increase with increasing electrolyte

FIGURE 9

The electrophoretic mobility of E. clathrata at different pH values. Each point represents the mean of 39 different measurements. Error bars indicate \pm standard deviation. See Materials and Methods for the buffers used.



concentration (Fig. 10). An increasing electrolyte concentration results in a decrease in the thickness of the electrical double layer (63). The algal cells were reversibly attached at lower concentrations of a divalent electrolyte (MgSO_4 , CaCl_2) than of a monovalent electrolyte (NaCl), an effect clearly related to the greater compression of the double-layer in the divalent electrolyte at comparable concentrations (63). However, in all three electrolyte systems, there were some algal cells still attached to the surface when the value of $1/K$ exceeded 200A. Cells attached to a hydrophobic substrate (polystyrene) did not respond to changes in electrolyte concentration.

Semi-reversible adhesion. As indicated in Figure 11, low electrolyte concentration had no effect on semi-reversible adhesion. There was no difference whether the electrolyte was divalent (MgSO_4 , CaCl_2) or monovalent (NaCl). The attachment of cells to glass by semi-reversible adhesion was not dependent on electrolyte concentration indicating a lack of dependence on the compression of the electrical double layer.

Irreversible adhesion. As in the case of semi-reversible adhesion electrolyte concentration had no effect on irreversible adhesion (Figure 12). It did not make any difference whether a divalent or monovalent electrolyte was used.

FIGURE 10

The theoretical double-layer thickness ($1/K$) and the % reversible adhesion at various concentrations of electrolyte. $1/K$ for NaCl ■-■ ; $1/K$ for CaCl_2 and MgSO_4 , ●-● ; % reversible adhesion in NaCl, ○-○ ; % reversible adhesion in CaCl_2 , □-□ ; % reversible adhesion in MgSO_4 , △-△ ; % reversible adhesion of cells attached to polystyrene in NaCl, ▲-▲ . The symbols represent the mean of 4 experiments with a standard deviation of ± 3 .

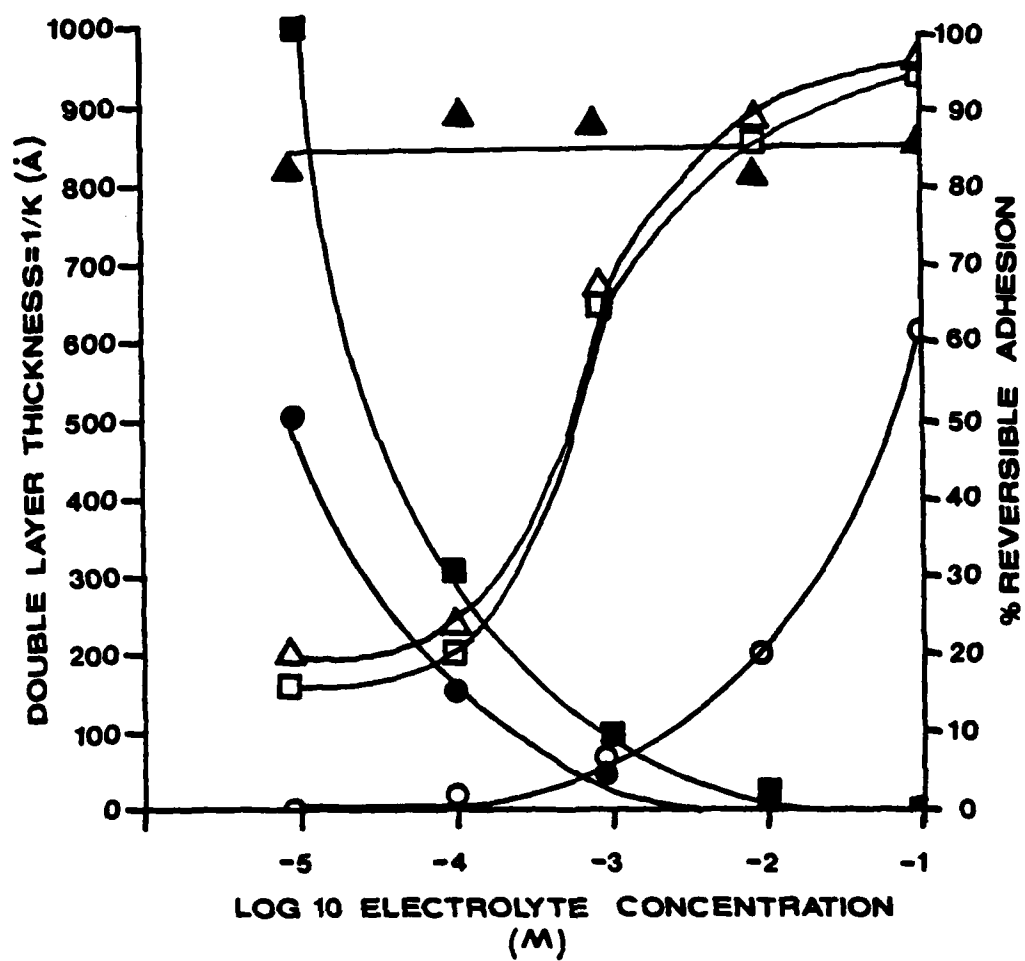


FIGURE 11

The effect of electrolyte concentration of monovalent and divalent cations on semi-reversible adhesion.

% adhesion in NaCl, ■-■ ; % adhesion in MgSO₄, ●-● ;

% adhesion in CaCl₂, ▲-▲ . Each point represents the mean of 4 experiments with a standard deviation of ± 3 .

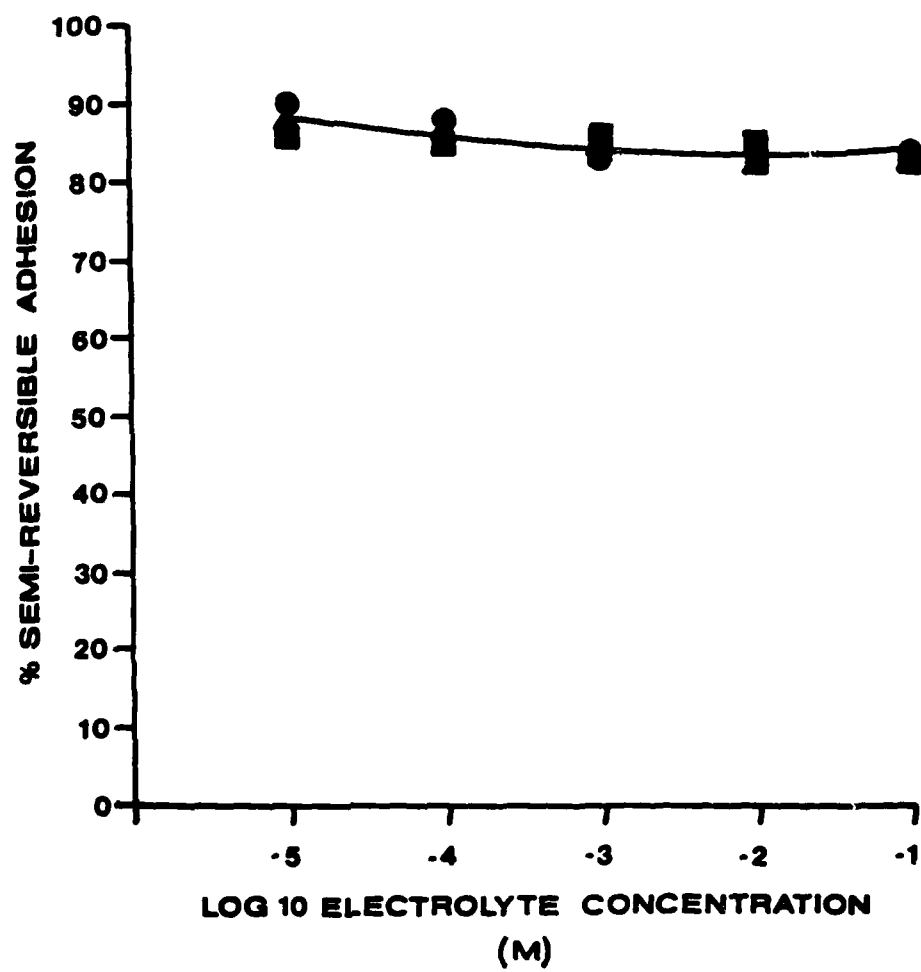
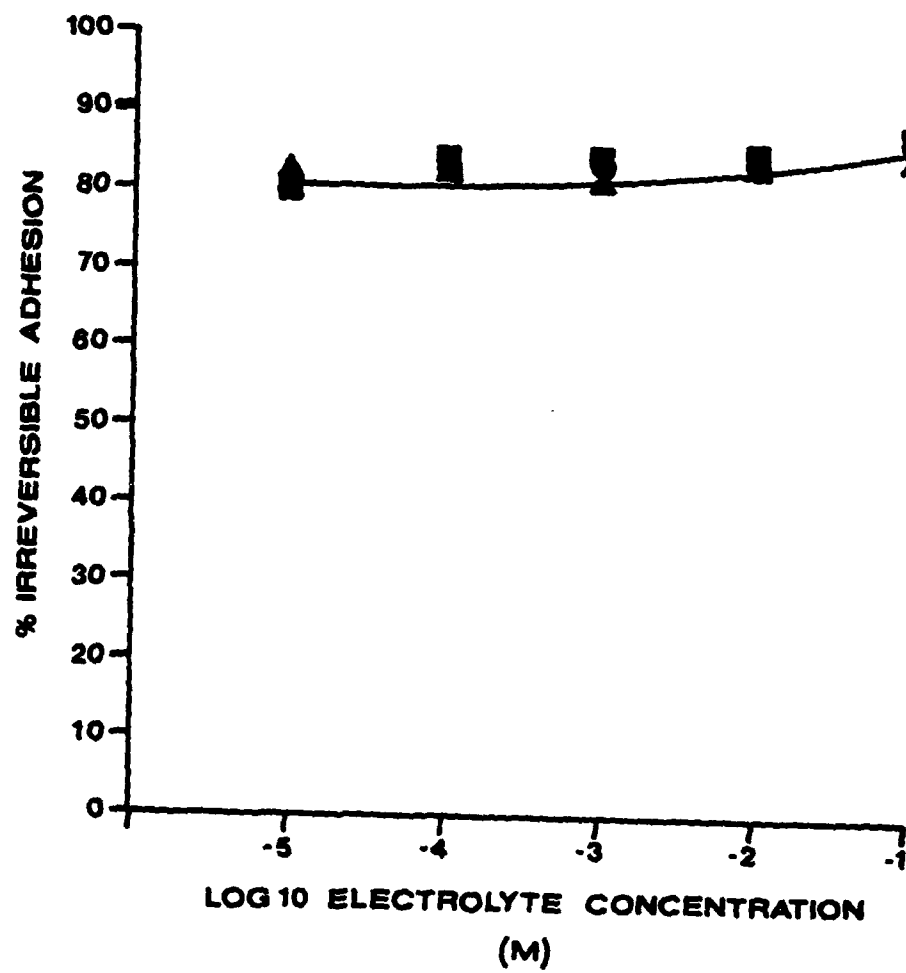


FIGURE 12

The effect of electrolyte concentration of monovalent and divalent cations on irreversible adhesion. % adhesion in NaCl, ■-■; % adhesion in MgSO_4 , ●-●; % adhesion in CaCl_2 , ▲-▲. The symbols represent the mean of 3 experiments with a standard deviation of ± 2 .



Hydrophobicity of Cells of Enteromorpha

To test the hydrophobicity of the cell surface of E. clathrata, cells were inoculated into a Sykes-Moore chamber filled with immersion oil and water (1:1). If cells disperse in the water phase they have a hydrophilic surface, whereas, if they disperse in the oil phase they have a hydrophobic surface. If they align at the interface, the cell surface has both hydrophilic and hydrophobic properties.

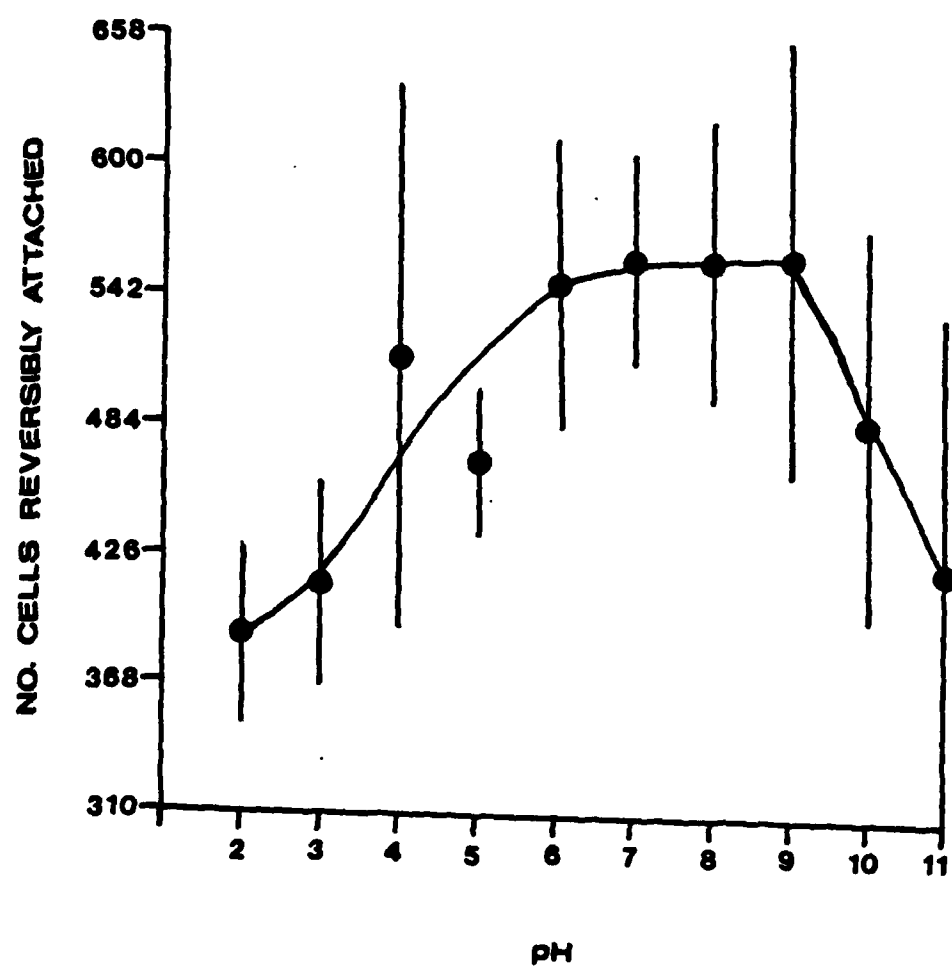
Cells of E. clathrata aligned themselves along the oil-water interface and in some cases entered into the oil phase. There was no particular orientation of the cells at the oil-water interface or in the oil phase.

Effect of pH on Adhesion

Reversible adhesion. As shown in Figure 13, pH had a limited effect on the reversible adhesion of cells of E. clathrata. From pH 6-9 the mean number of cells showing reversible adhesion was 560. At pH 2.0, approximately 30% fewer cells than at pH 6-9 became reversibly attached. Similarly, at pH 11.0 approximately 25% fewer cells than at pH 6-9 became reversibly attached. The large standard deviations were due primarily to the weak nature of reversible adhesion and to changes in the surface charge of both cell and substrate.

FIGURE 13

The effect of pH on reversible attachment of E.
clathrata to glass. Each point represents the mean of 6
different experiments. Error bars indicate \pm standard
deviation. See Materials and Methods for buffers used.
Each buffer also contained 2.5% NaCl.



Semi-reversible adhesion. External pH from 2-11 had no effect on semi-reversible adhesion (Fig. 14). Semi-reversible adhesion was established prior to introduction of the appropriate buffer. Semi-reversible adhesion was essentially constant at 90% over the entire range of pH values tested.

Irreversible adhesion. As shown in Figure 15, pH had a dramatic effect on the irreversible adhesion of E.

clathrata. Irreversible adhesion was established before introduction of the appropriate buffer. At lower pH values irreversible adhesion was reversed in that a stream of H₂O at 12 psi removed the cells from their glass substratum. As the pH increased, fewer cells were removed by the pressurized stream of H₂O. At pH 11.0, essentially maximal irreversible adhesion was maintained.

Cells treated with low pH (pH 5) buffer showed a dramatic difference in the nature of the extracellular material (Fig. 16B) as compared to untreated cells (Fig. 16A). The extracellular material of cells treated with pH 5 buffer became very diffuse and virtually disappeared.

Effect of Darkness and DCMU on pH

It was previously shown that prolonged darkness and treatment with DCMU caused a loss in irreversible adhesion of E. clathrata within 4 days (14). Another result of the incubation of E. clathrata in the dark or in 10^{-5} M DCMU was

FIGURE 14

The effect of pH on the semi-reversible adhesion of E. clathrata. Each point represents the mean of 5 different experiments. Error bars indicate \pm standard deviation. See Materials and Methods for the buffers used.

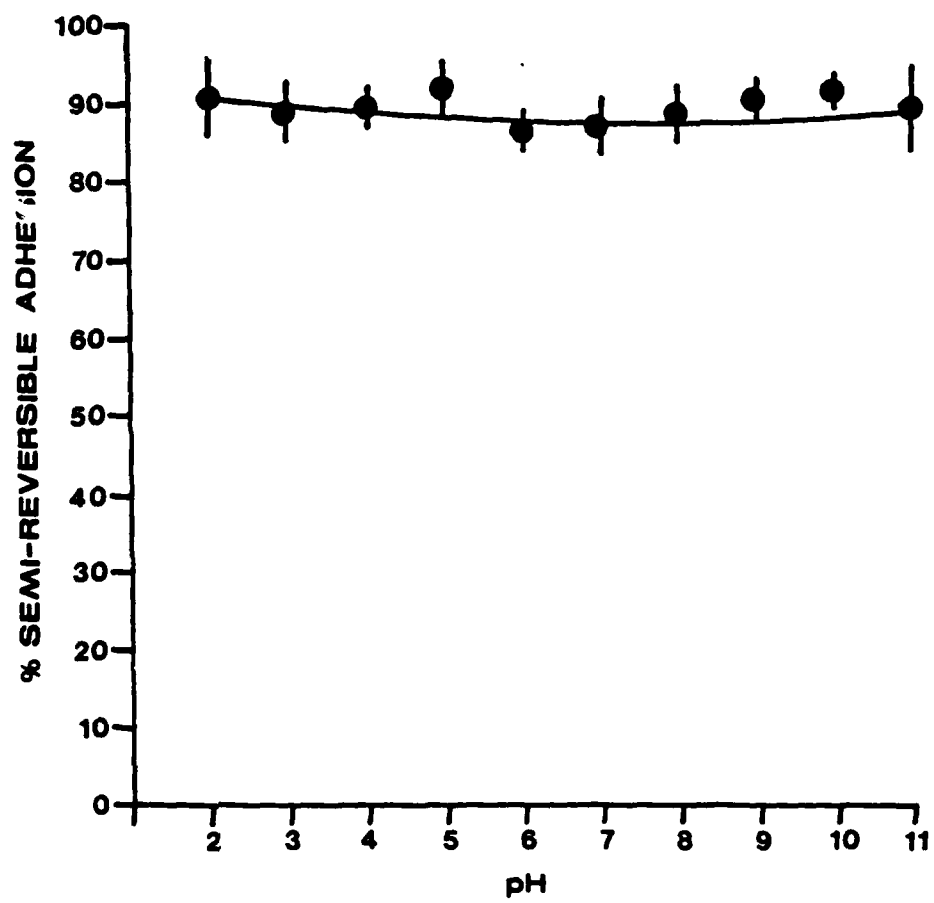
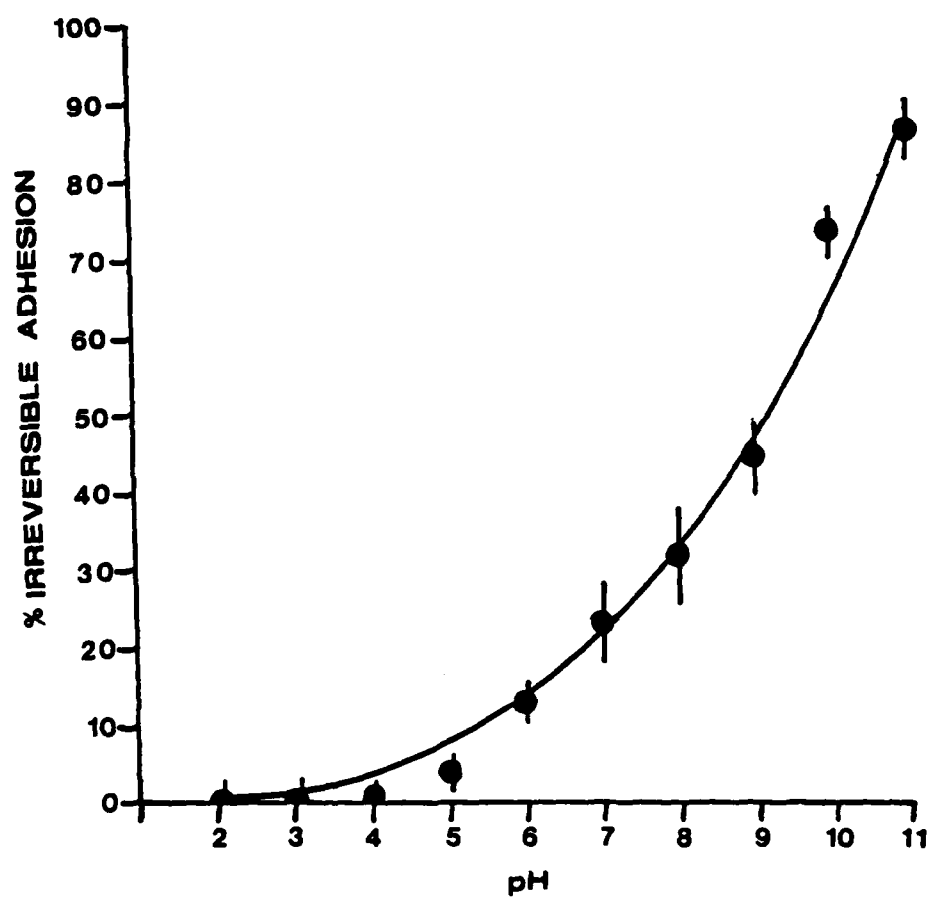


FIGURE 15

The effect of pH on the irreversible adhesion of E.
clathrata. Each point represents the mean of 11
experiments. Error bars indicate \pm standard deviation. See
Materials and Methods for the buffers used.



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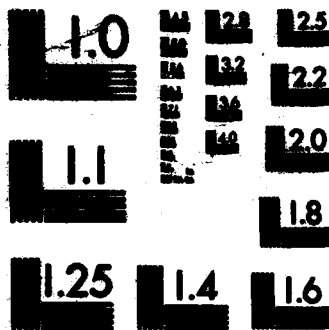
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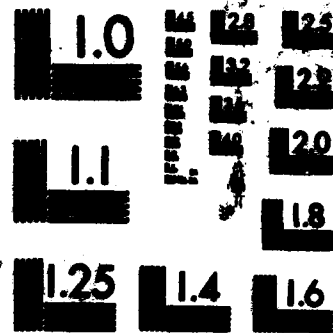
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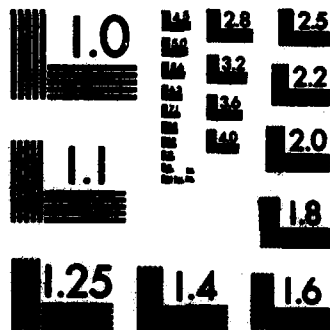
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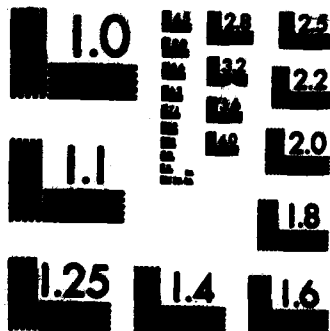
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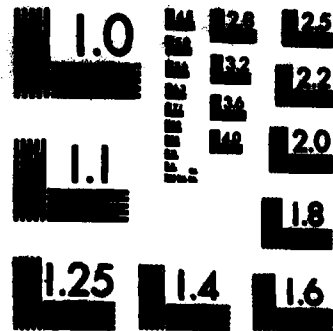
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FIGURE 16

The effect of pH 5 on cells of E. clathrata 14 days post inoculation as shown by transmission electron microscopy. A. Typical cell of E. clathrata that was attached to a substrate after 14 days of growth. The extracellular material (Ex) was present in large amounts and very dense. The extracellular material bridged the gap between the cell surface (CS) and substrate (S). This picture was representative of 15 separate viewings. Ruthenium red fixation. Bar marker represents 1.0 μm . B. The extracellular material (Ex) of the algal cell became diffuse and virtually absent when treated with a pH 5 buffer. This picture was representative of 15 separate viewings. There are artifact (Ar) holes present. Ruthenium red fixation. Bar marker represents 1.0 μm .



a decrease of the pH in the medium from 9.5 to 8.2 (Fig. 17 and 18). The pH dropped to 8.2 by the second day of incubation and then remained constant. The percent irreversible adhesion began a slow decline from zero time until day 2. At day 2, the loss in irreversible adhesion accelerated, reaching less than 10% by day 4.

Effect of Darkness on Irreversible Adhesion in a Continuous Flow of Medium A Buffered at pH 9.5

Since a drop in pH during prolonged darkness and a concomitant loss in irreversible adhesion was observed (see Fig. 18), I reasoned that if the drop in pH was responsible for the loss in irreversible adhesion then maintenance of constant pH during the dark treatment would prevent such a loss. As shown in Figure 19, irreversible adhesion of E. clathrata was not lost in the dark when the pH was kept near to 9.0 or above. Remarkably, even with continuously flowing medium buffered to pH 9.5 some acidification of the effluent occurred. The cause of this acidification is not presently known.

Effect of Growth on pH

During the growth of E. clathrata the pH of the culture was observed to change from an initial value of 7.7 to a

FIGURE 17

The effect of darkness on the pH of the medium and the subsequent effect on irreversible adhesion of E. clathrata. % adhesion, ●-●; pH, ■-■. Each point represents the mean of 5 experiments. Error bars indicate \pm standard deviation.

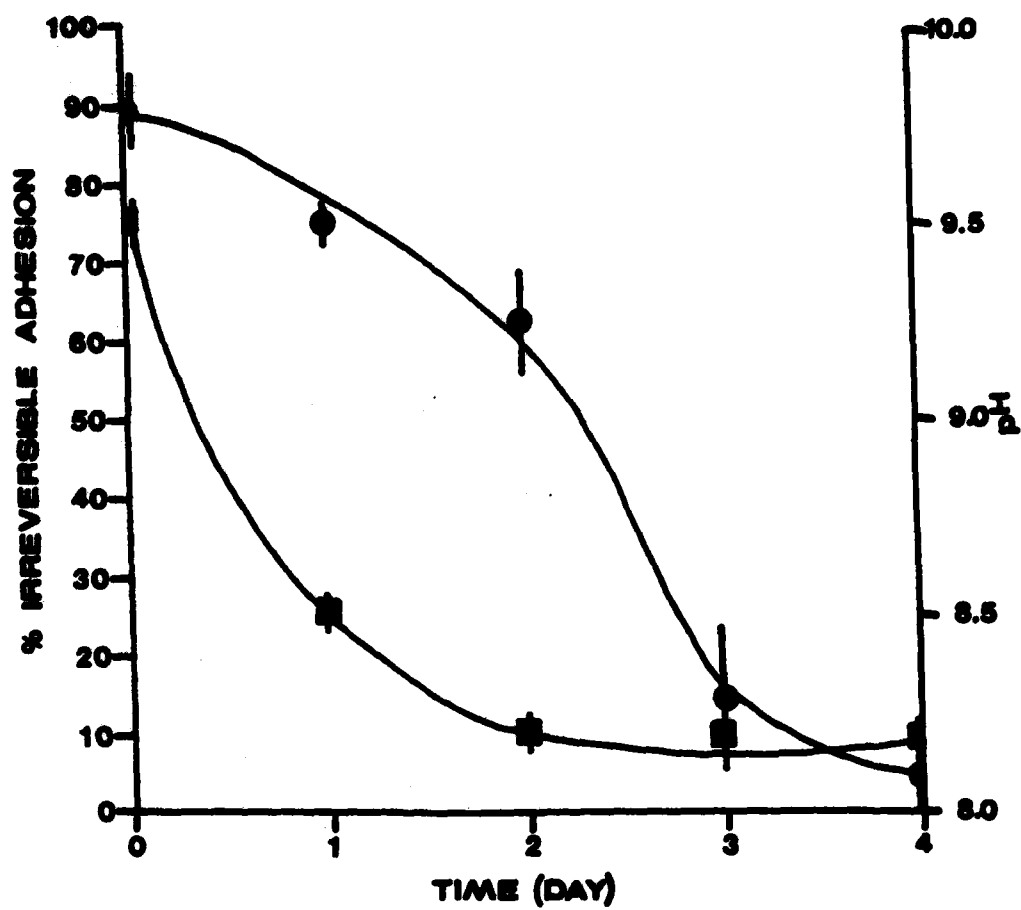


FIGURE 18

The effect of 10^{-5} M DCMU on the pH of the medium and the subsequent effect on irreversible adhesion of E. clathrata. % adhesion, ●-●; pH, ■-■. Each point represents the mean of 5 experiments. Error bars indicate \pm standard deviation.

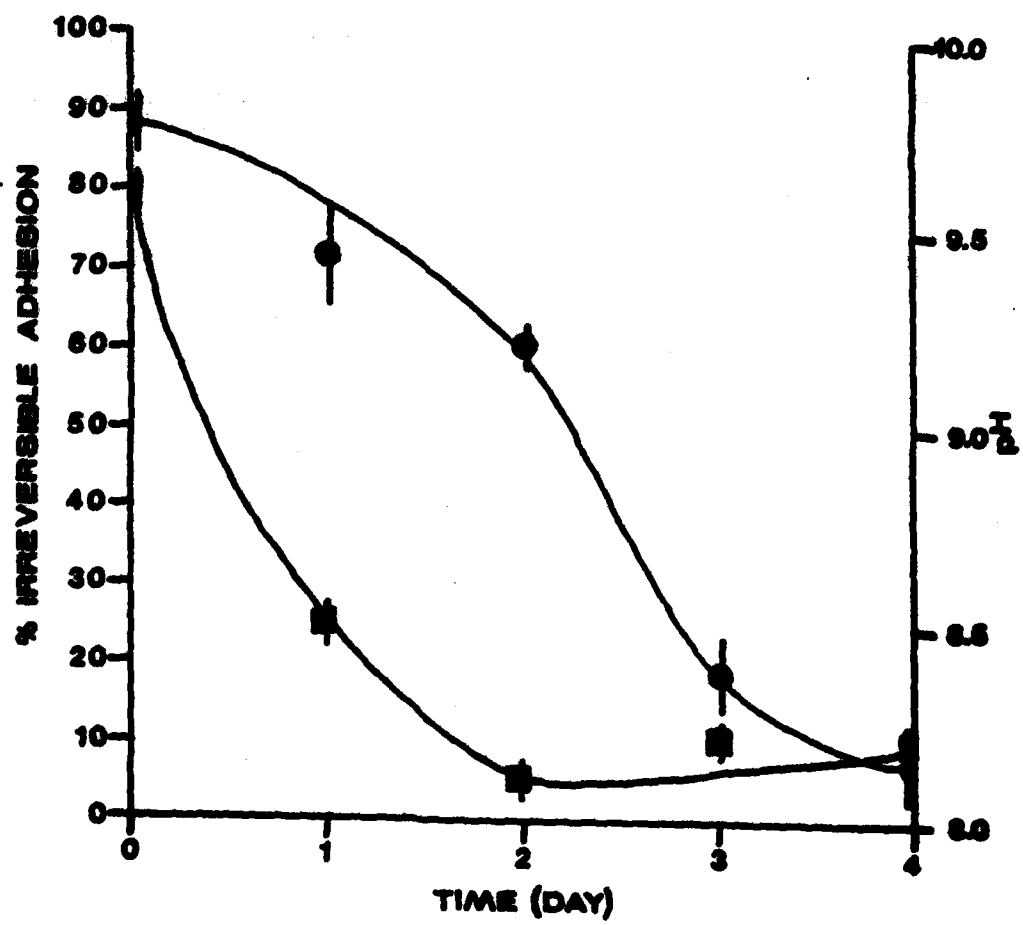
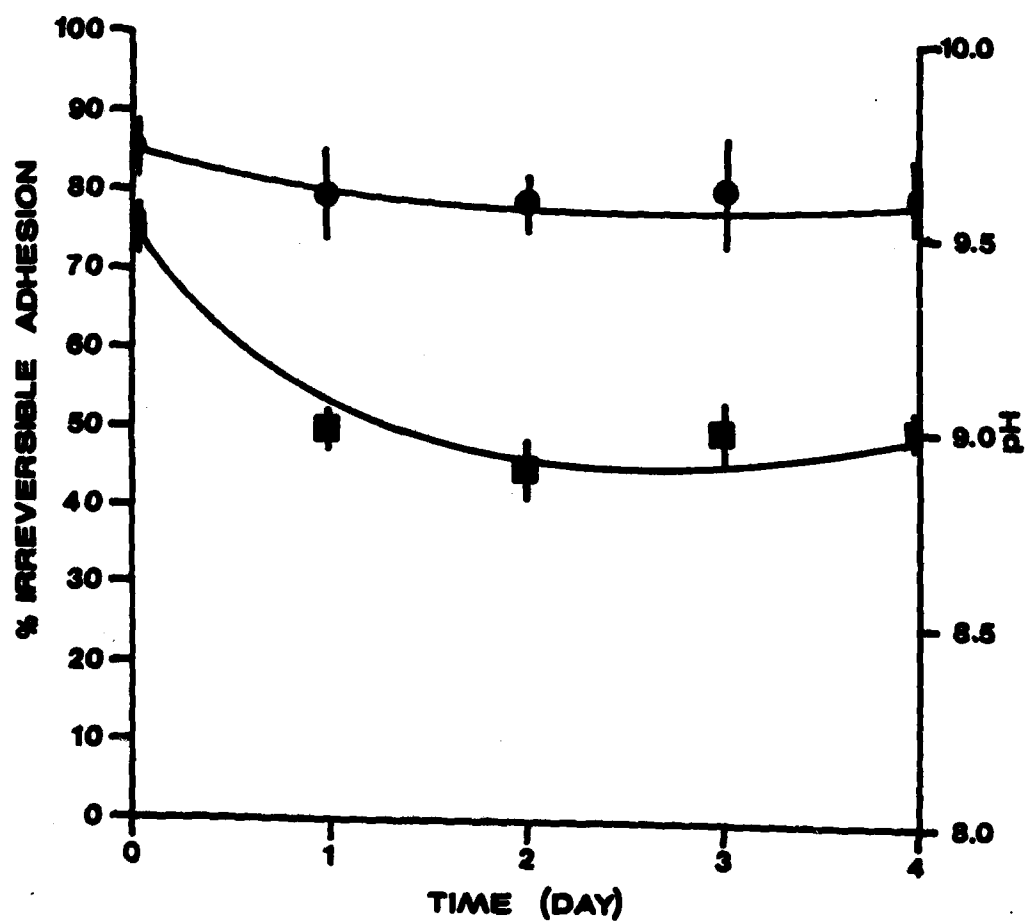


FIGURE 19

The effect of prolonged darkness on the irreversible adhesion of E. clathrata in a continuous flow of medium A buffered at pH 9.5. % irreversible adhesion, ●-●; pH, ■-■. Each point represents the mean of 5 experiments. Error bars indicate \pm standard deviation.



value greater than 9.5 at termination of the experiment (Fig. 20). Irreversible adhesion increased roughly with the increase in the pH of the medium, attaining maximal adhesion when the pH of the medium reached 9.0. If E. clathrata was grown in a continuous flow of medium A buffered at pH 7.7 a considerable reduction in percent irreversible adhesion was observed (Fig. 21). In the experiment shown in Figure 20, after 11 days of incubation the pH of the medium was 9.0 and the percent irreversible adhesion was 90, whereas as shown in Figure 21 after the same incubation period the pH of the medium was 7.9 and the percent irreversible adhesion was only 40.

Effect of Calcium on Irreversible Adhesion

Divalent cations such as calcium and magnesium have been shown to promote the attachment of marine bacteria. A marine Pseudomonad did not become firmly attached (irreversibly) in the absence of both cations, but did attach when either calcium or magnesium were present (63). Also, the initial reversible adhesion of an Achromobacter species has been shown to depend upon divalent cation concentration (63).

Previous work by the author (14) demonstrated that treatment of irreversibly adhered cells of E. clathrata with EDTA or HOEDTA resulted in the loss of irreversible adhesion. Another chelating agent, sodium pyrophosphate,

FIGURE 20

The change in the pH of the medium and in irreversible adhesion during the growth of E. clathrata. % irreversible adhesion, ●-●; pH, ■-■. Each point represents the mean of 5 experiments. Error bars indicate \pm standard deviation.

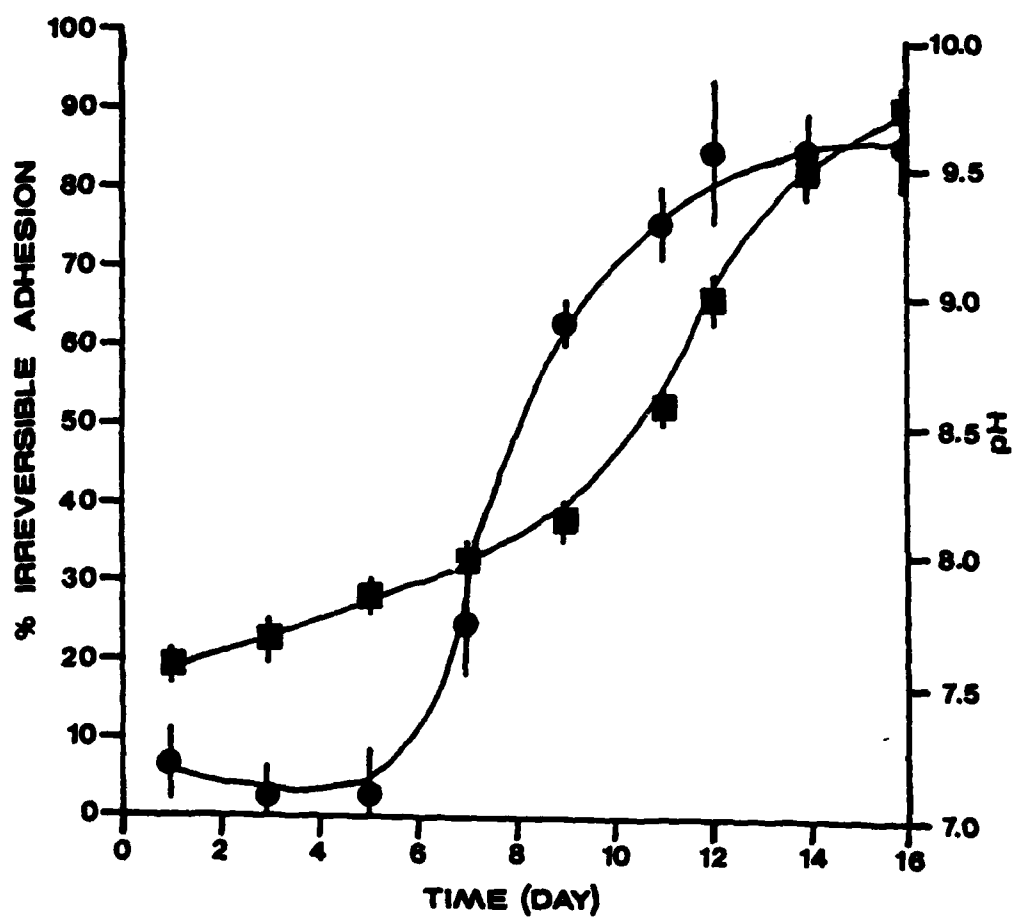
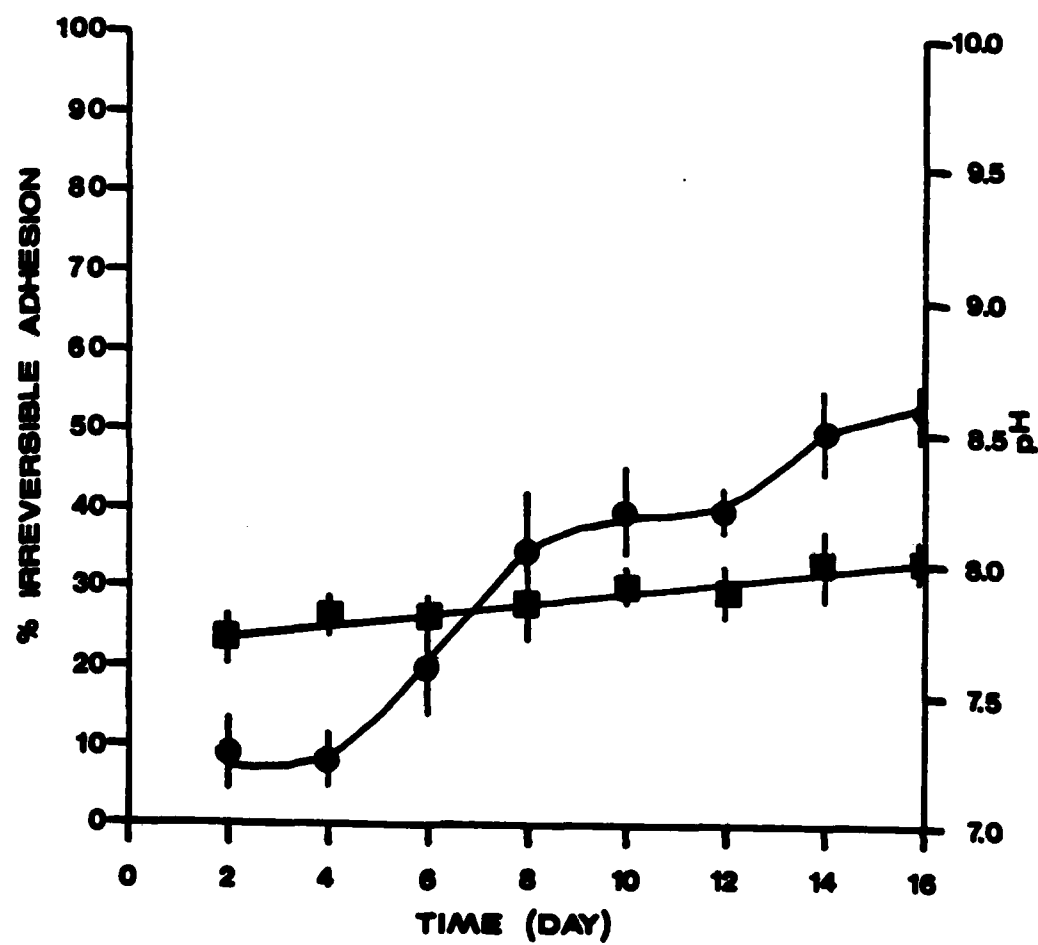


FIGURE 21

The effect of a continuous flow of medium buffered at pH 7.7 on the irreversible adhesion of E. clathrata. % irreversible adhesion, ●-● ; pH, ■-■ . Each point represents the mean of 5 experiments. Error bars indicate \pm standard deviation.

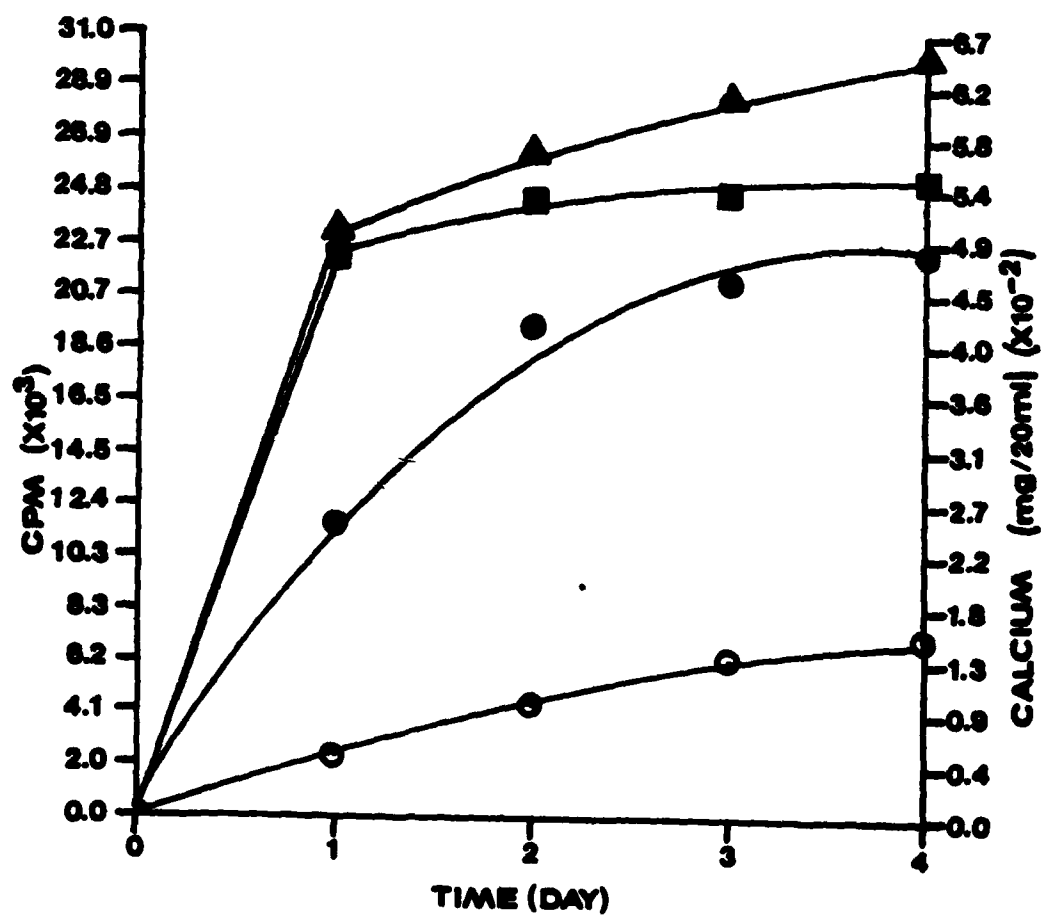


has been used to remove polyvalent cations which coordinate between soil minerals and organic polymers (84). Treatment with this sequestering agent (data not shown) caused a loss of irreversible adhesion similar to that observed in treatments with EDTA and HOEDTA.

Depletion of calcium from the culture medium also resulted in the progressive loss of irreversible adhesion with complete loss by the fourth day of incubation (14). Furthermore, if calcium was once again added to a calcium depleted culture irreversible adhesion was fully restored within 3 days (14). To further elucidate the role of calcium in the irreversible adhesion of E. clathrata, the following experiments were done. Cultures of E. clathrata were depleted of calcium by incubation without calcium under normal growth conditions for 4 days. Then cold calcium plus calcium-45 was added to the cultures which were incubated for an additional 4 days. At this point in the experiment, the culture medium was decanted and replaced with medium A, medium A minus calcium, medium A minus calcium plus 1 % EDTA or medium A minus calcium plus 1 % HOEDTA. The release of calcium from irreversibly adhered cells of E. clathrata was then monitored by determining the amount of calcium-45 in the culture fluid. The results are shown in Figure 22. There was an exchange of calcium in the adhesive material with the medium (medium A) amounting to 1.6×10^{-2} mg calcium/20 ml. There was a total of 4.8×10^{-2} mg calcium/20 ml released into the medium from cultures

FIGURE 22

The release of calcium into the medium following calcium removal treatments. Control, ○-○ ; medium A minus calcium, ●-● ; medium A minus calcium plus 1 % EDTA, ■-■ ; medium A minus calcium plus 1 % HOEDTA, ▲-▲ . The left vertical axis represents counts per minute of calcium-45 released into the medium while the right vertical axis represents the total calcium released into the medium. The symbols represent the mean of 5 experiments with a standard deviation of $\pm 0.2 \times 10^{-2}$ mg calcium/20ml.



incubated in medium A minus calcium. A further increase in the amount of calcium released into the medium amounting to 5.5×10^{-2} mg calcium/20 ml in medium A minus calcium plus 1 % EDTA and 6.5×10^{-2} mg calcium/20 ml in medium A minus calcium plus 1 % HOEDTA was observed.

When cells of E. clathrata were placed in the dark or treated with 10^{-5} M DCMU, a loss in irreversible adhesion was observed (reference 2 and Figs. 17 and 18 of the present study) which was due to a decrease in pH (Figs. 17 and 18). Cells of E. clathrata that had been labelled with calcium-45 in the manner described above were incubated in darkness or in 10^{-5} M DCMU and the release of calcium into the medium and the pH were determined. The results are shown in Figure 23. With both treatments a progressive release in calcium was observed. Release of calcium had essentially reached its maximum by the second day of incubation. The pH of the medium declined from 9.6 to 8.2 by day 2 and then flattened out.

To further clarify the effect of pH on calcium release, cells of E. clathrata were incubated in medium A adjusted to an initial pH of 8.0 or 5.0 with Tris-HCl (Fig. 24). In cultures with initial pH values of 8 or 5 there was an initial rapid release of calcium. However, by day 2 calcium was no longer being released, but was in fact, being re-absorbed. At the same time the measured pH of the culture was rising.

Algal cells treated with low pH or chelating agents

FIGURE 23

The release of calcium into the medium and subsequent drop in pH following prolonged darkness or the addition of 10^{-5} M DCMU. Prolonged darkness, ●-● ; 10^{-5} M DCMU, ○-○ ; measured pH of medium during prolonged darkness, □-□ ; measured pH of medium during incubation with 10^{-5} M DCMU, ■-■ . The right vertical axis represents the total amount of calcium released into the medium while the left vertical axis represents counts per minute of calcium-45 released into the medium. The symbols represent the mean of 5 experiments with a standard deviation of $\pm 0.3 \times 10^{-2}$ mg calcium/20 ml.

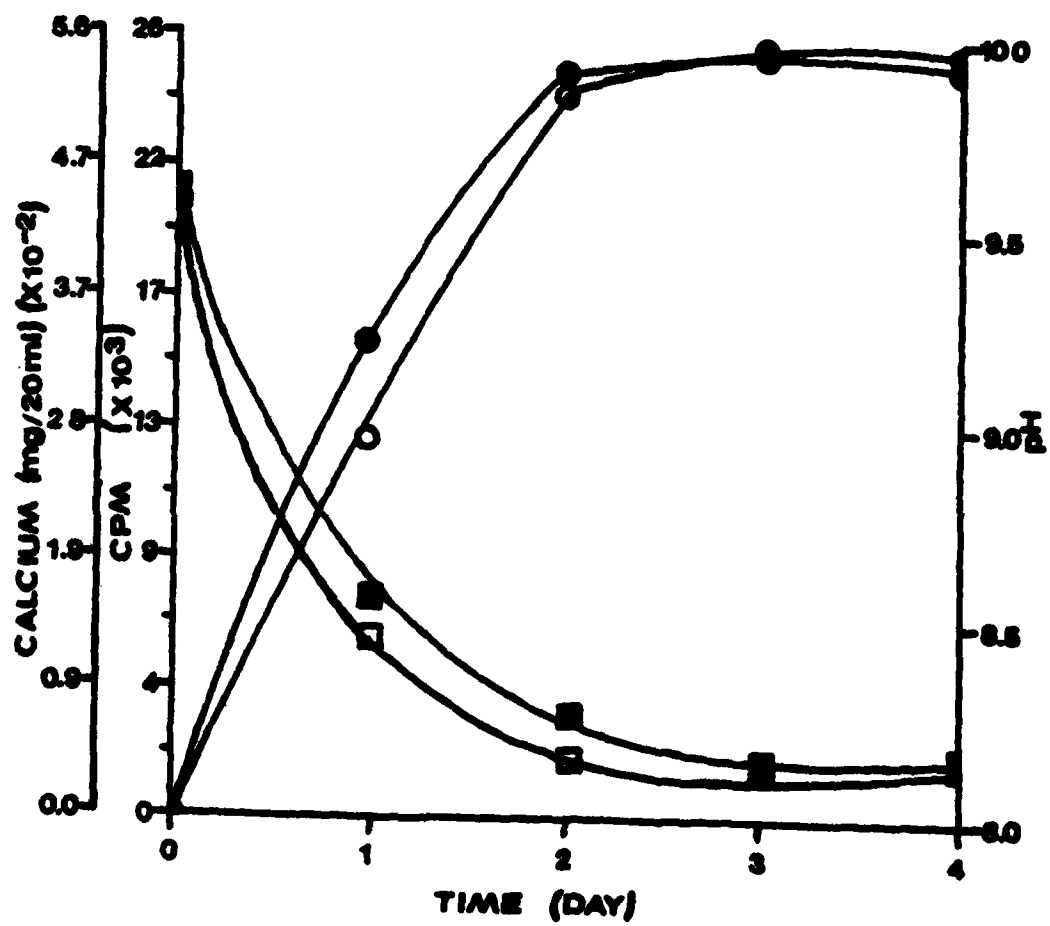
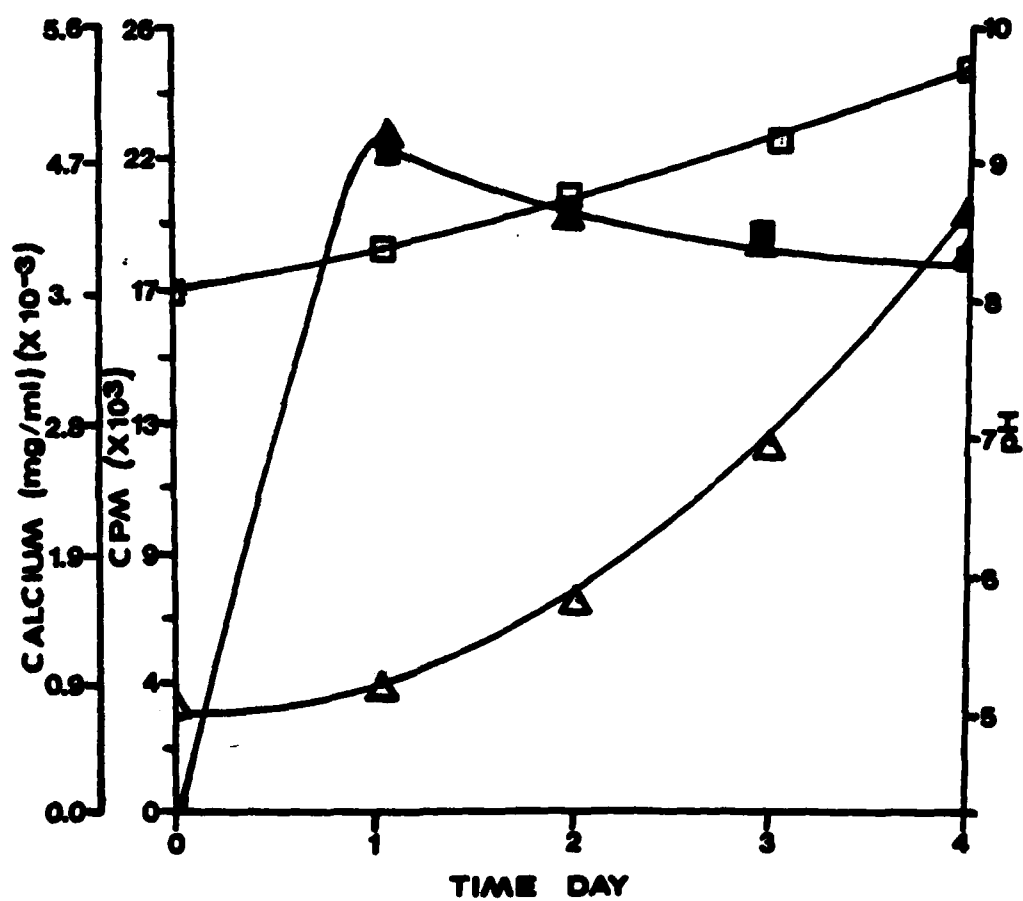


FIGURE 24

The release of calcium into the medium following a drop in pH and the subsequent rise in pH. Calcium release at pH 8, ■-■; calcium release at pH 5, ▲-▲; measured pH of culture medium initially at pH 8, □-□; measured pH of culture medium initially at pH 5, △-△. The right vertical axis represents the total amount of calcium released into the medium while the left vertical axis represents the counts per minute of calcium-45 released into the medium. The symbols represent the mean of 5 experiments with a standard deviation of $\pm 0.3 \times 10^{-2}$ mg calcium/20 ml.



showed a release of calcium and loss of irreversible adhesion (Figs. 22 and 24). Electron micrographs of cells treated with pH 5 buffer, 1 % EDTA, or 0.02 M pyrophosphate showed a noticeable loss in their extracellular material (Fig. 25B-D) when compared to untreated cells (Fig. 25A).

Effect of Chemical Treatment on Irreversible Adhesion

Sodium periodate and disodium tetraborate are known to denature polysaccharides and inhibit or disrupt cell adhesion (39). Glycol groups on the polysaccharides are oxidized by sodium periodate (84). The characteristic action of periodate was the oxidation and cleavage of adjacent hydroxyl groups found in sugars (39). Sodium periodate had a dramatic effect on the irreversible adhesion of E. clathrata (Table 4). Only 2 minutes of treatment with sodium periodate were required to reduce the irreversible adhesion to 5%. Disodium tetraborate reacts with adjacent cis hydroxyl groups of sugars resulting in negatively charged groups. These functional groups may react to form chemical cross-links among the carbohydrate molecules. Or, their mutual electrostatic repulsion may disrupt the parent carbohydrate (39). However, contrary to the results of Fletcher (39), disodium tetraborate had no disrupting effect on cell adhesion (Table 4).

FIGURE 25

Transmission electron microscopy of 14 day old cultures of E. clathrata that have undergone various chemical treatments. A. Untreated cell of E. clathrata showing large amounts of extracellular material (Ex) which bridged the gap between cell surface (CS) and substrate(S). This picture was representative of 15 separate viewings. Ruthenium red fixation. Bar marker represents 1.0 um. B. Cells treated with a pH 5 buffer. There was a noticable loss in the extracellular material (Ex). This picture was representative of 15 separate viewings. Ruthenium red fixation. Bar marker represents 1.0 um. C. Cells treated with 1% EDTA. Again there was a loss in amount and compactness of extracellular material (Ex). This picture was representative of 15 separate viewings. Ruthenium red fixation. Bar marker represents 1.0 um. D. Cells treated with pyrophosphate showed a loss of extracellular material. This picture was representative of 15 separate viewings. Ruthenium red fixation. Bar marker represents 1.0 um.



Table 4

The Effect of Chemical Treatments on Irreversible Adhesion¹

Treatment	% Adhesion
Control	85
Sodium Periodate ²	5
Disodium Tetraborate ³	92

¹Based on the mean of 5 experiments with a standard deviation of ± 6 .

²Sodium periodate was at a concentration of 0.02 M.

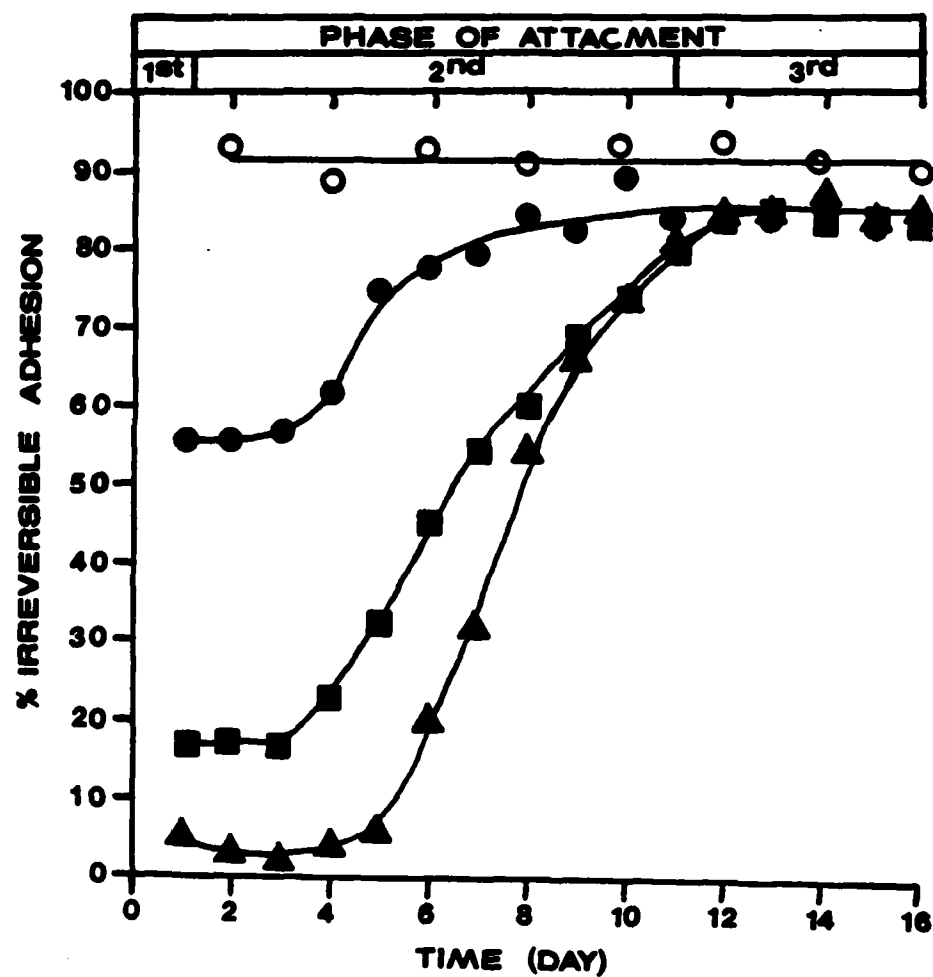
³Disodium Tetraborate was at a concentration of 0.02 M.

Effect of Enzyme Treatment

Preliminary experiments indicated that trypsin, protease, and α -amylase partially hydrolysed (within 12 hours) the adhesive material of 14 day old cultures of E. clathrata. The effects of these enzymes were very slight, causing a loss of adhesion from 83 percent to 63 percent (14). Trypsin is a proteolytic enzyme that hydrolyses peptide bonds only at the carboxyl group of the basic amino acids L-arginine and L-lysine. Protease is a general proteolytic enzyme that attacks peptide bonds in a wide range of amino acids. Amylase hydrolyses polysaccharides with α 1,4 glucosidic bonds (29). These three enzymes were used to determine their effect on adhesion during the three phases of attachment. As stated in Materials and Methods culture flasks were inoculated with E. clathrata at zero time. The appropriate enzyme was added to a culture on day 1 and incubation continued until day 16. On day 2 enzyme was added to a 2 day old culture and incubation continued until day 16. A similar procedure was used each day until termination of the experiment at day 16. The fact that some cultures exhibited a loss of adhesion when incubated with an enzyme for 15 days while other cultures incubated with an enzyme for 12 hours did not, does not mean these enzymes needed a long incubation period to hydrolyze the adhesive material (Fig. 26). As stated above these enzymes reached their maxium effect within 12 hours and longer incubation

FIGURE 26

The effect of the enzymes α -amylase, trypsin, and protease on adhesion of E. clathrata during the 3 phases of attachment. Irreversible adhesion with α -amylase, ●-●; irreversible adhesion with trypsin, ■-■; irreversible adhesion with protease, ▲-▲; irreversible adhesion without enzyme (control), ○-○. The time the cultures are in one of the three phases of adhesion is indicated at the top of the graph. The standard deviation was $\pm 10\%$ for 5 separate experiments.



did not increase the loss of adhesion.

All three enzymes exhibited their maximum effect during the end of the first phase of attachment (reversible adhesion) and the early part of the second phase of attachment (semi-reversible adhesion) (Fig. 26). Protease had the strongest effect on adhesion, trypsin the next strongest, and -amylase had the least effect. As the cultures advanced through the second phase of attachment and started entering into the third phase of attachment (irreversible adhesion) the enzymes exhibited less and less of an effect. When cultures were in the third phase of attachment the effect of the enzymes were very slight as compared to the control (Fig. 26).

Chemical Analysis of the Adhesive Material

If the adhesive material binding the cell surface to a substrate can be sufficiently weakened, then the cells can be removed intact with some of the adhesive material remaining on the substrate. Removal of calcium from the medium for a period of 4 days was shown to cause a loss in irreversible adhesion (14). Calcium deprivation was used as an initial step in the isolation of the extracellular material responsible for adhesion. By the criterion of visual inspection all cellular material was removed by scraping the glass surface with a rubber policeman after calcium deprivation. The phenol-H₂O extraction procedure of

Westphal and Jann (92) has been used to extract lipopolysaccharides and glycoproteins from cells. This method was used to extract any remaining extracellular material from the glass surface.

Cell wall of E. clathrata was purified by the procedure of Dodson and Aronson (32). The purity of the isolated cell walls was determined by microscopic examination of I₂KI stained walls. The phenol-H₂O extraction procedure (92) was performed on the cell walls to extract any loosely-bound material.

Phenol-H₂O extraction of the glass flasks yielded a small amount of material (464.20 ug). The extracted material partitioned into the aqueous phase. Thus, only the aqueous phase of the phenol-H₂O extracted cell wall was used so that a direct comparison could be made with the isolated adhesive material.

To elucidate whether the material extracted from the glass surface (adhesive material) was contaminated with cell wall, both phenol-H₂O extracted samples were analyzed with descending paper chromatography. The extracted material from cell wall produced 3 spots while only one spot was produced from the extracted glass surface (Fig. 27). All 4 spots stained for both carbohydrate and protein. The mobilities of the single spot from the adhesive material was different from the 3 spots generated from the cell wall. Gross chemical analysis for carbohydrate, protein, and uronic acid of the phenol-extracted material of the cell

FIGURE 27

Tracing of a descending paper chromatogram of phenol-H₂O extracted material from the cell wall of E. clathrata and from the glass substrate. Whatman No. 1 paper with n-butanol:pyridine:water (6:4:3) solvent. The solvent was allowed to run off the bottom of the chromatogram.

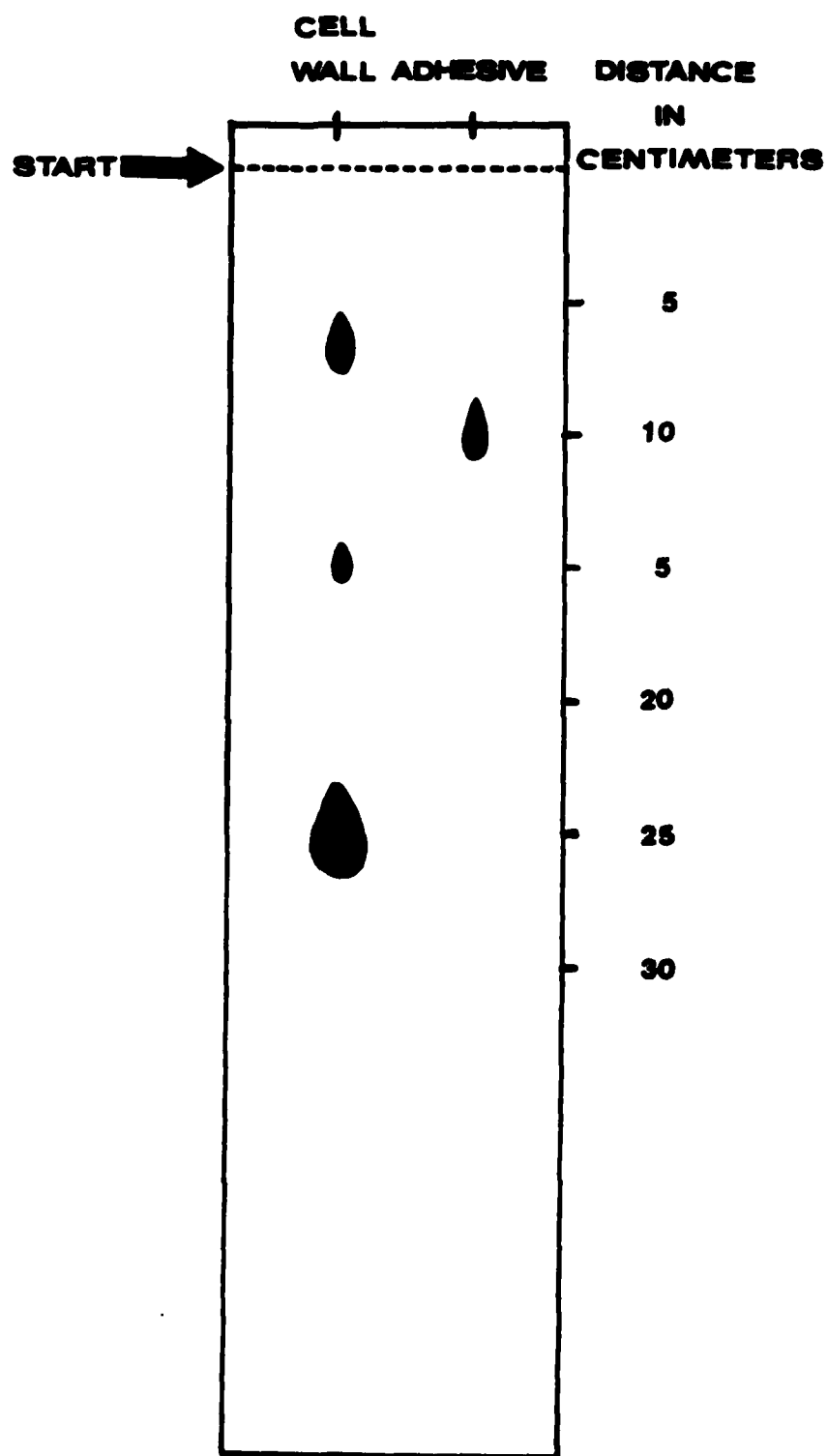


TABLE 5

Analysis of Carbohydrate, Protein, and Uronic Acid from the Aqueous Phase of the Phenol-Water Extraction of Cell Wall and Adhesive Material.

	Total Material	Protein	Carbohydrate	Uronic Acid
	μg	μg	μg	μg
Adhesive	116.05	6.66	152.54	6.85
Cell Wall	122.82	5.37	164.18	15.27

wall and the adhesive material are shown in Table 5.

The carbohydrate assay used was qualitative rather than quantitative because it over estimates the amount of carbohydrate present (33). The assays used for protein and uronic acid determination were quantitative. Thus, the values for protein and uronic acid presented in Table 5 can be taken at face value whereas the value for carbohydrate is only a rough estimate.

With the use of gas chromatography it was possible to elucidate the chemical nature of the carbohydrate portion of the adhesive material and cell wall (Table 6). The ratio of the various sugars in the cell wall and adhesive material did not match. Fucose and galactose were found in higher concentrations in the adhesive material than in the cell wall, while glucose was much higher in the cell wall. Glucosamine was found only in the cell wall. The chemical analysis of the cell wall of E. clathrata corresponded closely with the results reported by Dodson and Aronson (32) for E. intestinalis with the exception that xylose was not detected in E. clathrata while fucose was detected. The carbohydrate to protein ratio of the adhesive material was approximately 23:1 while the ratio in the cell wall was 31:1.

TABLE 6

Chemical Composition of the Carbohydrate Portion from
the Aqueous Phase of the Phenol-Water Extraction of Cell
Wall and Adhesive Material.

	Fucose	Galactose	Glucose	Glucosamine	Total
	μg	μg	μg	μg	μg
Adhesive	13.78	1.597	87.741	—	103.1
Cell Wall	7.5	0.73	95.7	7.5	110.7

DISCUSSION

In 1935 Zobell and Allen (97) showed that once a bacterium was attached to a surface, firm attachment required several hours of incubation. Since then, studies on attachment of bacteria to a substrate have confirmed with few exceptions (38) two phases of attachment. Marshall et al.(63) termed these two phases reversible and irreversible sorption.

The attachment of cells of E. clathrata was similar to that of bacteria in that it exhibited a reversible and an irreversible stage. However, the attachment of E. clathrata differed from marine bacterial attachment (63) in that it took 11 days for irreversible adhesion to become established while attachment of bacteria takes place between 2 to 48 hours. Another difference in the attachment of E. clathrata and that of bacteria lies in the demonstration of a discrete intermediate stage between reversible adhesion and irreversible adhesion. I have termed this stage semi-reversible adhesion because it differed from reversible adhesion, but was not strong enough to be termed irreversible adhesion.

The three phases of adhesion were: 1) reversible adhesion in which the cells were held very weakly at the surface. Cells were removed from a surface when rinsed in medium A, 2.5% NaCl, or distilled water. The cells also exhibited Brownian motion; 2) semi-reversible adhesion, in

which the cells were weakly attached to the surface. Cells remained on a surface when rinsed in medium A, 2.5% NaCl or distilled water, but were removed when washed under low pressure with medium A or distilled water. Cells did not exhibit Brownian motion; and 3) irreversible adhesion in which the cells were very strongly attached to the surface. Distilled water at a pressure of 12 psi did not remove attached cells nor did they exhibit Brownian motion.

Four factors must be considered in the determination of the mechanism of attachment of cells of E. clathrata: 1) the surface of the algal cells; 2) the surface of the substrate; 3) the medium separating the algal surface from that of the substrate; and 4) environmental factors which may affect any or all of the preceding (39).

When cells of E. clathrata were inoculated into a flask containing an aqueous phase (Medium A) they settled to the bottom due to gravity. Settling was a passive process since the cells used were non-motile. As the cells settled onto a glass surface, they became subject to short-range attractive forces which were capable of weakly holding the cells at the solid-liquid interface (60). The cells were readily removed from the surface at this stage by rinsing with distilled water. Thus, the term reversible adhesion is used to describe this situation. Reversible adhesion took place in approximately 6 hours (Fig. 2). The extended time period required to establish reversible adhesion was probably due to the large radius of curvature of the cells which

diminishes the local strength of the attractive forces (91). Reversible adhesion was passive in that no biological activity on the part of the cells was required. This was demonstrated by the fact that cells killed by UV irradiation (Fig. 6) still became reversibly attached. UV irradiation kills cells by inactivating DNA, but so far as it is known, UV irradiation causes no change in the cell wall. If attachment depends on the integrity of the cell wall, it will not be affected by UV-induced death (65). Thus, reversible adhesion is strictly a physicochemical process.

There are several types of processes which could be responsible for reversible attachment of cells in aqueous media: 1) the DLVO theory of lyophobic colloid stability (81); 2) hydrophobic bonding (61); 3) electrostatic interactions (59); or 4) specific reactions between surface functional groups (30). E. clathrata cells had a constant negative surface charge, as shown by its constant negative mobility over the pH range 2-11 (Fig. 9). This indicated that on the algal cell surface there were ionogenic groups which were exclusively acidic. Enteromorpha species typically have carboxyl sulfate esters which account for 16.8 % of the cell dry weight (76). Since there was essentially no change in electrophoretic mobility from pH 2 to 11, carboxyl sulphate ester groups were probably the predominate ionogenic group involved (76). Since carboxyl sulphate esters are strongly acidic ($pK=1.0$), this could explain why the cells did not exhibit zero mobility or

charge reversal in mobility at pH 2.0 as is common with bacteria. Further, the negative mobility at pH 2.0 indicated that there were few amphoteric groups present. The absence of amphoteric groups has also been observed with the unicellular green alga, Chlorella (68). Other negatively charged groups that could be present are phosphatidic groups (46).

Glass is a hydrophilic material, in that it has ionogenic functional groups (41) and becomes more negatively charged due to the dissociation $\text{-SiOH} \rightleftharpoons \text{-SiO}^- + \text{H}^+$ as the pH is increased (68).

Cells of E. clathrata were probably attracted to surfaces of like charge such as with glass under conditions where van der Waals attraction energies exceeded the electrical double-layer repulsion energies. This may be explained in terms of the DLVO theory (59,81,90). The DLVO theory, as modified by Shaw (81), involves an estimation of the London-van der Waals attractive energies between two surfaces and the electrical repulsion energies resulting from the overlapping ionic atmospheres (diffuse double layer) around the surfaces (63,81). At decreasing electrolyte concentration (increasing values of $1/K$ which is the thickness of the electrical double layer) there is an increase in the magnitude of the resultant repulsive forces which causes an increase in particle separation or expulsion of cells from the surface. At high electrolyte concentration cells become close enough to the surface to

enter into the secondary potential energy minimum (attractive trough) (44,59,81) and become reversibly attached.

When E. ciathrata cells were sorbed reversibly at a surface, they were not anchored in any way to the surface: that is, cells were not at the primary potential energy minimum (area of molecular contact) (44). However, E. ciathrata cells were attracted to the secondary minimum, which is at a small but finite distance from the glass surface. Reversible sorption of cells to a surface can be overcome by the application of some force such as simple rinsing or by reducing the electrolyte concentration to a point where the organisms are repelled from the surface. This was shown to be the case for E. ciathrata cells (Fig. 10). Thus, reversible adhesion to glass was probably due to a balance between the van der Waal attractive and repulsive forces. The initial attachment of Chlorella has also been explained by the DLVO theory (68). Moreover, the reversible stage of bacterial attachment has been explained in a similar way (45,59,63,78,91).

Since glass has a hydrophilic surface (see Table 2) and E. ciathrata has a hydrophilic surface (see Fig. 10 and discussion above), hydrophobic bonding does not play a part in reversible adhesion to glass. However, it does play a role in reversible adhesion to such surfaces as polystyrene which is hydrophobic. In the case of polystyrene electrolyte concentration had no effect (Fig. 10).

Strong electrostatic attractive forces can exist between heterogeneous surfaces (68). However, electrostatic forces were probably not involved in the reversible adhesion of E. clathrata to glass, since both the algal and glass surfaces were negatively charged. Moreover, electrostatic interactions are not dependent on electrolyte concentration, but adhesion was dependent on electrolyte concentration (Fig. 10).

The effect of pH on reversible adhesion (Fig. 13) was very slight. The slight effect in the lower pH range of 2-5 was due to a decrease in the net negative surface charge on the algal cell surface as shown by the decrease in electrophoretic mobility in this range (Fig. 9). The drop in adhesion in the upper pH range of 10-11 was again probably due to changes in the net negative surface charge of the cell and has been shown to occur with some bacteria (68) but lacks explanation.

The properties of the substrate can have a large effect on attachment (28). The events occurring at the liquid-surface interface within the first few hours are critical and are the most likely to be influenced by the surface properties of the substrate. Attachment has been shown to be influenced by surface wettability (31,3) and by surface free energy (41,39). Several types of forces contribute to the free energy of a surface, and these include dispersion, dipole, electrostatic, and metallic forces (39).

The nature and packing of the exposed groups of atoms

at any surface determine its wettability, a characteristic feature generally independent of the nature and arrangements of the underlying atoms and molecules (2). The critical surface tension is often used as an index of surface wettability. As the critical surface tension increases, so does wettability. It has been shown that a critical surface tension of approximately 25 dynes/sq.cm corresponded to a minimum level of attachment (2). Greater levels of attachment occurred on substrates with values of surface tension both above and below 25 dynes/sq.cm. The greater the surface charge the more hydrophilic the substrate. Conversely, the lower in surface charge the more hydrophobic the substrate. In most cases, bacteria either attach to hydrophobic surfaces or to hydrophilic surfaces, but not to both. The critical surface tensions of the substrates used to study the adhesion of E. clathrata ranged from 33 dynes/sq.cm to greater than 74 dynes/sq.cm, thus, going from low wettability to high wettability. Both hydrophobic and hydrophilic substrates as well as organic and synthetic polymers were tested (Tables 2 and 3). E. clathrata demonstrated the ability to attach to all surfaces tested. This indicates another difference between the attachment of E. clathrata and bacteria.

One of the most important observations about biological adhesion in relevant natural circumstances is that it rarely occurs without the mediation of preadsorbed films of biological macromolecules, primarily glycoproteins and

proteoglycans (4). High energy substrates (such as glass) undergo a dramatic decrease in critical surface tension within seconds due to adsorption of bio-films (88), but do not become hydrophobic (31). Fletcher (36) found a relationship between attachment and the concentration of dissolved organic material in the medium. Marine organisms attached more quickly and tightly when the surface was first covered with organic material (96). It has also been suggested that these primary films insulate organisms from toxic substrates (64). The isoelectric point of a protein will determine whether it is positively charged or negatively charged at the pH of the medium. The adsorption of proteins of various isoelectric points on to a glass surface had no effect on the irreversible adhesion of E. clathrata. Thus, unlike bacteria (4) and the green alga Chlorella (87) the attachment of E. clathrata was independent of preadsorbed organic films.

Reversible adhesion, due to its easily reversible nature and non specificity of substrate (Table 2,3) rules out any covalent interactions between surface functional groups. The effect of electrolyte concentration and lack of any pH effect on reversible adhesion (Fig. 13) also makes this type of interaction unlikely. Taken as a whole, the data indicate that E. clathrata demonstrates a high degree of versatility in its attachment to a surface.

Approximately 30 hours after reversible adhesion took place, cells of E. clathrata entered into the second stage

of attachment called semi-reversible adhesion (Fig. 3). Semi-reversible adhesion differs from reversible adhesion in that cells were not removed by gentle rinsing in distilled water, but were removed with a low pressure stream of distilled water. Also, cells in the semi-reversible stage of adhesion did not exhibit Brownian motion. Semi-reversible adhesion was an active process in that it required biological activity. UV killed cells were not able to progress from reversible to semi-reversible adhesion (Fig. 7). The biological activity required to establish semi-reversible attachment was the synthesis of an extracellular fibrous material. A series of EM pictures (Fig. 5) showed that an extracellular fibrous material was present 35 hours after inoculation, but not before. This material was present through out the remainder of growth. The production of this extracellular material would allow cells to bridge the repulsion barrier (see discussion of DLVO theory above) and become held to the surface at the point of molecular contact (primary potential energy minimum) (15,44). The loss in dependence on electrolyte concentration (Fig. 11) showed that this stage of adhesion was no longer dependent on the compression of the electrical double-layer, and attraction at the secondary minimum. It has been demonstrated in a wide range of bacteria that the production of extracellular polymer was responsible for adhesion to a substrate (21,23,26,39,40,42,59,62,63,74,78, 86,91). The adhesion of Chlorella vulgaris may also depend

on the synthesis of extracellular polymers (87).

Feldtman and McPhee (35) showed that the energy involved in physical adsorption between two solid phases separated by a fluid phase was more than adequate to produce adhesive forces greater than the cohesive strength of either substrate or cell. It is not necessary that any specific chemical affinities exist for adhesion of materials, but the extracellular polymer must have good wettability to insure close molecular contact of the two surfaces if physical forces are to suffice (2). Materials that are not viscous can insure good wetting of a surface (15). When two solid surfaces are separated by a thin layer of liquid which wets them both well, adhesion results. Such a thin fluid phase between two surfaces would join them together, with the joint having some tensile strength but only such resistance to shear as is determined by the viscosity of the liquid film. Semi-reversible attachment was very susceptible to shear force, suggesting that the extracellular fibrous material had low viscosity. An extracellular material composed of single strands of protein and carbohydrate chains with no cross-linking would produce a material with very low viscosity, but excellent wettability. This would produce an adhesive with tensile strength but very little shear strength. The lack of cross-linking, especially of the ionic bond type, would explain why pH had no effect on semi-reversible adhesion (Fig. 14).

Approximately 11 days after inoculation, cells of E.

clathrata entered into the third stage of attachment termed irreversible adhesion (Fig. 4). This stage differs from the first two in that very strong adhesion to a substrate was exhibited. E. clathrata was attached so strongly to glass that a water pressure of 12 lb/sq.in. would not dislodge it. Irreversible adhesion, like semi-reversible adhesion, was not dependent on electrolyte concentration (Fig. 12) and, thus, not dependent on compression of the electrical double-layer.

However, like reversible adhesion, this stage of adhesion was passive since cells killed with UV irradiation at the mid-point of the semi-reversible phase of adhesion were capable of irreversible adhesion as long as the pH of the medium was 9.5 (Fig. 8). If the pH of the medium was below 8.5 irreversible adhesion did not take place (Fig. 8). It has been shown that pH had a dramatic effect on this stage of adhesion (Fig. 15). The ability of dead cells to enter into irreversible adhesion suggested that some type of physicochemical process was occurring. This could have been a pH dependent modification of the extracellular material that was already present. Irreversible adhesion was at its maximum by day 11. When the cells were inoculated into medium A the pH was 7.7 (Fig. 20). As the cells grew, the pH of the medium increased to 9.6 by day 12 (Fig. 20). Conversely, if the pH was kept constant at 7.7-8.0 then irreversible adhesion never occurred (Fig. 21).

The modification taking place in the extracellular

material (adhesive material) was most likely a hardening or curing process. An analogy to this would be the hardening or curing of epoxy adhesives. This curing or hardening of extracellular material to produce irreversible adhesion has been alluded to in some bacterial systems and was thought to be a result of cross-linking of the extracellular polymer chains by divalent cations (2). The permanence of an adhesive joint can be influenced by an increase in viscosity of the adhesive layer through permeation and formation of cross-links (2). The pH will determine the degree of protonation of ionogenic groups associated with the extracellular material and thus determine the formation of ionic cross-linking (47).

It has been suggested that calcium and other multivalent ions increase the viscosity, thus, increasing the shear strength of the adhesive material by forming these cross-links in the extracellular polymer (2,19,63,91). Calcium will cause an increase in viscosity of a solution of alginic acid which is made up of uronic acid residues (48). Calcium has already been shown to be specifically required for irreversible adhesion (14). By using a radioisotope of calcium (calcium-45) it was shown that calcium was bound into the adhesive polymer (Fig. 22). If calcium was involved in building cross-links among the adhesive polymer chains, and this was pH dependent, then, lowering the pH should result in a release of the calcium from the adhesive polymer. A lowering of the pH to 8 or 5 resulted in the

release of calcium (Fig. 24). This was extracellular calcium since there was no further release of calcium when EDTA was added. Treatment of cells with EDTA or HOEDTA resulted in the sequestering of calcium from the extracellular material (Fig. 22).

Thus, the mechanism for the formation of irreversible adhesion was dependent on the formation of cross-links between the polymer chains. These cross-links were due to ion-triplet formations with calcium. In order for formation of these cross-links, the pH of the medium must be approximately 9-9.5.

Other environmental factors that affect irreversible adhesion are prolonged darkness and DCMU (14). I have demonstrated that prolonged darkness and treatment with DCMU resulted in a drop in pH from 9.6 to 8.2 and a loss in irreversible adhesion (Fig. 17,18). Calcium was also lost from the extracellular material (Fig. 23). Thus, darkness and DCMU affect irreversible adhesion by lowering the pH which in turn leads to a loss in calcium from the extracellular material.

A lot of work has been done in trying to elucidate the chemical composition of the adhesive material that has been implicated in irreversible adhesion. One way to gain information about the chemistry of the adhesive material is to study the effect of enzyme treatment on adhered cells. Once the cells had entered the phase of irreversible adhesion there was essentially no effect by the enzymes

used. However, cells that were in reversible and semi-reversible adhesion were affected by all three enzymes. This suggested that the adhesive material was composed of both carbohydrate and protein. The fact that these enzymes lost their effectiveness as the cells entered the irreversible phase of adhesion supports the notion that the adhesive material had undergone a curing or hardening reaction. As more cross-links were formed, the less accessible the susceptible bonds became to enzymatic attack.

Information can also be gained by chemically treating attached cells to see if attachment is consequently lost. It was shown that sodium periodate (Table 4) had a severe effect on adhesion. Disruption of attachment by periodate again suggested that a carbohydrate adhesive was involved since the characteristic action of periodate is the oxidation and cleavage of adjacent hydroxyl groups found in sugars.

Treatment with borate can also cause removal of attached bacteria (39). Borate reacts with the adjacent hydroxyl groups of sugars to give negatively charged groups (95) which could enter into cross-linkage reactions. However, borate had no effect on the adhesion of E. clathrata (Table 4). Because cross-linking was important in the curing of the adhesive material, the action of borate might possibly be enhancement of cross-linking. Pyrophosphate also affected the adhesion of E. clathrata (data not shown) in the same manner that EDTA affected it

(Fig. 22) (14).

EDTA and pyrophosphate are sequestering agents that are used to remove polyvalent cations from biological material. Hence, EDTA and pyrophosphate removed calcium from the adhesive material and, thus, caused a loss of irreversible adhesion.

By far the most accurate determination of the makeup of a biological material is through chemical analysis. The adhesive material of E. clathrata was composed of protein (6%) and carbohydrate (89%). There is also approximately 6% uronic acid present (Table 5). Calcium has been shown to bind specifically to the carboxyl groups of glucuronate (19,72). Calcium also binds to uncharged carbohydrates, especially fucose which is a common terminal residue of the oligosaccharide chains on glycoproteins (19). The sugars making up the carbohydrate portion (Table 6) are commonly found in algae. Glucose, galactose, fucose, and uronic acid are also common constituents of extracellular material produced by film-forming marine bacteria (22). Even though the adhesive material of E. clathrata was not an integral part of the cell wall, part of it could still be bound or enmeshed among the cell wall polymers.

In this study E. clathrata was shown to go through three phases of attachment. These phases are reviewed in Figure 28. The first phase (reversible adhesion) was the initial attachment or attraction of E. clathrata cells to a surface. This was a passive process involving various types

FIGURE 28

A Summary of the Three Phases of Attachment

0	REVERSIBLE ADHESION	Suceptible to Ionic Concentration
	(DLVO)	Suceptible to 2.5% NaCl Rinse
		Suceptible to Shear Force
		Not Suceptible to pH
1		Does Not Require Calcium
2	SEMI-REVERSIBLE	Suceptible to Shear Force
3	ADHESION	Not suceptible to Ionic
4	(Synthesis	concentration
5	of Extracell-	Not suceptible to 2.5% NaCl
6	ular material)	Rinse
7		Not suceptible to pH
8		Does Not Require Calcium
11	IRREVERSIBLE	Suceptible to pH
12	ADHESION	Not Suceptible to Shear Force
13	(Cross-	Not Suceptible to 2.5% NaCl
14	linking of	Rinse
15	extracell-	Not Suceptible to Ionic
16	ular material)	Concentration
17		Requires Calcium

of attractive forces. The type of attractive force was dependent on the surface. When the surface was hydrophilic the attractive force was the van der Waal force. If the surface was hydrophobic then attraction was by hydrophobic interaction. Reversible adhesion was highly susceptible to ionic concentration (except for hydrophobic attachment) and any type of externally applied force.

The second phase (semi-reversible) which was unique to E. clathrata was an active one where extracellular polymer was synthesized and laid down between the algal cell wall and the surface. The polymer acted as an adhesive attaching E. clathrata to a surface. The extracellular polymer was composed of carbohydrate, protein, and uronic acid residues. Semi-reversible adhesion was not susceptible to ionic concentration nor to gentle rinsing. However, E. clathrata cells were easily removed when any shear force was applied.

The third phase (irreversible adhesion) was a passive phase during which cross-links between the extracellular polymer and calcium formed. The most probable site for these cross-linking ionic bonds were between uronic acid groups in the polymer chain. This phase was very susceptible to pH (below 9.0). A slight drop in pH caused dissociation of the calcium. The irreversible phase of attachment was very strong and cells of the rhizoidal filaments remained attached even after scrubbing. The ability of E. clathrata to withstand extremes in the environment, its regenerative powers, and its strong attachment make this alga the prime

fouler of large ships.

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